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(54) Titre : POLYPEPTIDES POSSEDANT UNE ACTIVITE DE TRANSPORTEUR D'ACIDE DICARBOXYLIQUE EN C4 ET
POLYNUCLEOTIDES CODANT POUR CES DERNIERS
(54) Title: ASPERGILLUS ACULEATUS DERIVED POLYPEPTIDES HAVING C4-DICARBOXYLIC ACID TRANSPORTER ACTIVITY
AND POLYNUCLEOTIDES ENCODING SAME

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      M L G Q H P P P D T S C S D L T T Y Q H
1 ATGCTCGGGC AACATCCGCC TCCCGACACC TCCTGCTCGG ACCTTACAAC ATACCAGCAT
  E L K A S K Y S S S T N V S L R D R L R
61 GAGGTCAAAG CCTCCAAATA CTCTAGTTCC ACCAATGTGT CTCTACGGGA CCGTCTGCGT
  H F T W A W Y T L T M S T G G L A L L L
121 CATTTTACCT GGGCGTGTA TACTCTGACT ATGAGCACCG GCGGTCTAGC CCTCCTGCTG
  A S Q P Y S F S G L Q Q I G L A V Y I I
181 CCCAGCCACC CCTACTCCTT CTCCGGACTG CAACAGATCG GGCTTGCAGT CTACATCATC
  N L A F F A L L C S L N A A R F I L H G
241 AACCTGGCCT TCTTTGCGTT GCTGTGTAGC CTCATGGCCG CACGCTTCAT TCTCCACGGC
  N F L D S L R H D R E G L F F P T F W L
301 AACTTCCCTG ACTCCCTCCG ACACGACCCG GAGGGTCTTT TCTTCCCTAC TTTCTGGCTT
  S I A T I I T G L Y R Y F G D T T Q P A
361 TCTATTGCAA CTATCATCAC CGGCCTGTAC CGCTACTTCG GCGACACCAC ACAGCCTGCA
  F I Y A L E V L F W L Y C A F T L M T A
421 TTCATTTACG CTCTTGAGGT GCTCTTCTGG CTCTACTGTG CCTTCACTCT GATGACCGCT
  I I Q Y S F V F T A H H Y P L Q T M M P
481 ATTATCCAAT ACTCCTTTGT CTTTACCGCC CACCACTACC CTCTACAAAC GATGATGCCC
  S W I L P A F P I M L S G T I A S V I A
541 TCATGGATCC TCCCGCATT CCTATCATG CTCAGCGGCA CGATCGCCTC CGTCATTGCC
  E Q Q P A R S A I P M I V A G T T F Q G
601 GAACAGCAGC CCGCGCGCTC TGCTATTCCC ATGATCGTCG CCGGCACCAC CTTCCAAGGC
  L G F S I S F L M Y A H Y I G R L M E T
661 CTGGCTTCT CCATCAGTTT CCTCATGTAC GCGCACTATA TCGGGCGGCT CATGGAGACG
  G L F S R E H R P G M F I C V G P P A F
721 GGCCTTCCGT CCCGGGAACA CCGAOCGGG ATGTTTATCT GCGTTGGCCC CCCGGCTTTC
  T A L A L I G M T N G L P E D F Q V L Q
781 ACGGCCCTTG CCCTAATCGG CATGACCAAC GGCCTTCCTG AGGATTTTCA AGTCCTTCAA
  D P H P F Q D A H I L R L L A I A T G A
841 GACCCGCACC CTTTCAAGA CGCGCACATC CTCCGACTCC TTGCCATCGC CACGGGCGCC
  F L W A L S L W F F S I A I I A T I R L
901 TTCCCTCTGGG CCCTCAGTCT CTGGTTCTTT AGCATTGCCA TCATCGCCAC CATCCGCCTC
  F P T A F H L N W W A M V F P N T G F T
961 CCACCTACAG CCTTCCACCT CAACTGGTGG GCCATGGTTT TTCCAAACAC GGGTTTACT
  L A T I T L G K A F D S P G V K G V G S
1021 CTCGCGACCA TCACGCTGGG CAAAGCCTTC GATAGCCCTG GAGTCAAGGG CGTCGGATCT
  A M S I C I V G M W L F V F A S N I R A
1081 GCCATGTCCA TTTGCATCGT GGGGATGTGG CTGTTCTGTT TTGCGAGCAA TATCCGTGCC
  V V K R D I V F P G K D E D V S E *
1141 GTTGTCAAAC GGGATATTGT TTTCCCTGGG AAGGACGAGG ATGTATCGGA GTAA
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(57) Abrégé/Abstract:
The present invention relates to isolated polypeptides having C4-dicarboxylic acid transporter activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides, and methods of producing C4-dicarboxylic acids, such as malic acid.

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(71) Applicant (for all designated States except US):
NOVOZYMES, INC. [US/US]; 1445 Drew Avenue,
Davis, California 95618 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **FISCHER, Aman-
da** [US/US]; 3310 Arroyo Court, Davis, California 95618
(US). **YAYER, Debbie** [US/US]; 5632 Hoag Place,
Davis, California 95618 (US).

(74) Agent: **FECHTER, Eric**; Novozymes, Inc., 1445 Drew
Avenue, Davis, California 95618 (US).

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[Continued on next page]

(54) Title: ASPERGILLUS ACULEATUS DERIVED POLYPEPTIDES HAVING C4-DICARBOXYLIC ACID TRANS-
PORTER ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

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      M L G Q H P P P D T S C S D L T T Y Q H
1  ATGCTCGGGC AACATCCGCC TCCCGACACC TCCTGCTGGG ACCTTAGAAC ATACCAGCAT
   E L K A S K Y S S S T N V S L R D R L R
61  GAGCTCAAAG CCTCCAAATA CTCTAGTTCC ACCAATGTGT CTCTACGGGA CCGTCTGCGT
   H F T W A W Y T L T M S T G G L A L L L
121 CATTTACCT GGGCGTGGTA TACTCTGACT ATGAGCACCG GCGGTCTAGC CCTCCTGCTG
   A S Q P Y S F S G L Q Q I G L A V Y I I
181 GCCAGCCAGC CCTACTCCTT CTCCGACTG CAACAGATCG GCGTTGCAGT CTACATCATC
   N L A F F A L L C S L M A A R F I L H G
241 AACCTGGCCT TCTTTGCGTT GCTGTGTAGC CTCATGGCCG CAGCCTTCAT TCTCCACGGC
   N F L D S L R H D R E G L F F E P T F W L
301 AACTTCCTCG ACTCCCTCCG ACACGACCGC GAGGGTCTTT TCTTTCCTAC TTTCTGGCTT
   S I A T I I T G L Y R Y F G D T T Q P A
361 TCTATTGCAA CTATCATCAC CGGCCTGTAC CGTACTTCG GCGACACCAC ACAGCCTGCA
   F I Y A L E V L F W L Y C A F T L M T A
421 TTCATTACG CTCTGAGGT GCTCTCTGG CTCTACTGTG CTTCACTCT GATGACCGCT
   I I Q Y S F V F T A R H Y P L Q T M M P
481 ATTATCCAAT ACTCCTTTGT CTTTACCGCC CACCACTACC CTCTACAAAC GATGATGCCC
   S W I L P A F P I M L S G T I A S V I A
541 TCATGGATCC TCCCGCATT CCCTATCATG CTCAGCGGCA CGATCGCCTC CGTCATTGCC
   E Q Q P A R S A I P M I V A G T T F Q G
601 GAACAGCAGC CCGCGCGCTC TGCTATTCCC ATGATCGTCG CCGGCACCAC CTTCCAAGGC
   L G F S I S F L M Y A H Y I G R L M E T
661 CTGGGCTTCT CCATCAGTTT CCTCATGTAC GCGCACTATA TCGGGCGGCT CATGGAGACG
   G L P S R E H R P G M F I C V G P P A F
721 GGCCTTCCGT CCGGGGAACA CCGACCCGGG ATGTTTCTCT GCGTTGGCCC CCGGCTTTTC
   T A L A L I G M T N G L P E D F Q V L Q
781 ACGGCCCTTG CCTAATCGG CATGACCAAC GGCCTTCTCG AGGATTTTCA AGTCCCTCAA
   D P H P F Q D A H I L R L L A I A T G A
841 GACCCGCAAC CCTTTCAAGA CGGCACATC CTCCGACTCC TTGCCATGCG CACGGGCGCC
   F L W A L S L W F F S I A I I A T I R L
901 TTCCTCTGGG CCTCAGTCT CTGGTTCTTT AGCATTGCCA TCATCGCCAC CATCCGCCTC
   P P T A F H L N W W A M V F P N T G F T
961 CCACCTACAG CCTTCCACCT CAACTGGTGG GCCATGGTTT TTCCAAACAC GGGTTTACT
   L A T I T L G K A F D S P G V K G V G S
1021 CTCGCGACCA TCACGCTGGG CAAAGCCTTC GATAGCCCTG GAGTCAAGGG CGTCGGATCT
   A M S I C I V G M W L F V F A S N I R A
1081 GGCATGTCCA TTGTCATCGT GGGGATGTGG CTGTTCTGTG TTGCGAGCAA TATCCGTGCC
   V V K R D I V F P G K D E D V S E *
1141 GTTGTCAAC GGGATATTGT TTTCCCTGGG AAGGACGAGG ATGTATCGGA GTAA

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Fig. 3

(57) Abstract: The present invention relates to isolated polypeptides having C4-dicarboxylic acid transporter activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides, and methods of producing C4-dicarboxylic acids, such as malic acid.

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5 petrochemical feedstocks due to lower production costs. However, the rising cost of petroleum derived building block chemicals, the geopolitical instability affecting crude oil prices, and the desire to implement manufacturing processes that utilize feedstocks derived from renewable resources have stimulated a renewed interest in producing organic acids and other chemicals by microbial fermentation.

While malic acid is produced commercially today by chemical synthesis from petrochemical feedstocks, it can also be produced by microbial fermentation. Malic acid has been produced at high levels in genetically engineered yeast (*Saccharomyces cerevisiae*) (Zelle et al., 2008, *Appl. Environ. Microbiol.* 74: 2766-2777) and naturally occurring
10 filamentous fungi such as *Aspergillus* spp. (U.S. Patent No. 3,063,910; Bercovitz et al., 1990, *Appl. Environ. Microbiol.* 56: 1594-1597). Abe et al. (U.S. Patent No. 3,063,910) and Bercovitz et al. (1990, *Appl. Environ. Microbiol.* 56: 1594-1597) reported high levels of malic acid production in several species of *Aspergillus*. Moreover, Battat et al. (1991, *Biotechnol. Bioengineering*, 37: 1108-1116) reported malic acid production as high as 113 g/L by
15 *Aspergillus flavus* in a stirred fermentor under optimized conditions. Dicarboxylic acid production by microbial fermentation in yeast is described in WO 2010/003728. Malic acid production by microbial fermentation is also described in WO 2008/011974 and WO 2009/155382. Improvement of malic acid production by genetic engineering of *Aspergillus* will enable economical commercial malic acid production by fermentation.

20 Malic acid overproduction in *Aspergillus* spp. occurs under specific culture conditions (aerobic conditions and high C:N ratio; calcium carbonate is also added as a neutralizing agent and as source of CO₂ for malic acid biosynthesis). Under these conditions, overflow metabolism via the cytosolic, reductive tricarboxylic acid (TCA) cycle results in increased malic acid biosynthesis and secretion into the culture medium. Increased malic acid
25 production has been reported in *Saccharomyces cerevisiae* by increasing the level of pyruvate carboxylase (Bauer et al., 1999, *FEMS Microbiol Lett.* 179: 107-113) or malate dehydrogenase (Pines et al., 1997, *Appl. Microbiol. Biotechnol.* 48: 248-255) using genetic engineering and increasing expression of a malic acid transporter (Zelle et al., 2008, *supra*). It has been suggested, based on biochemical evidence, that malate dehydrogenase activity
30 is limiting malic acid production in *Aspergillus flavus* strain A TCC 13697 (Peleg et al., 1988, *Appl. Microbiol. Biotechnol.* 28: 69-75). PCT Publication No. WO2011/028643, entitled "Methods for Improving Malic Acid Production in Filamentous Fungi" filed August 27, 2010, describes malic acid production in filamentous fungi.

It would be advantageous in the art to improve C4-dicarboxylic acid production, such
35 as malic acid production, in *Aspergillus* as a result of genetic engineering using recombinant DNA techniques. The present invention provides, *inter alia*, polypeptides having C4-

dicarboxylic acid transporter activity, polynucleotides encoding the polypeptides, and methods for improving C4-dicarboxylic acid production (e.g., malic acid production).

Summary of the Invention

5 The present invention relates to isolated polypeptides having C4-dicarboxylic acid transporter activity. In one aspect the isolated polypeptides having C4-dicarboxylic acid transporter activity are selected from: (a) a polypeptide having at least 65% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions
10 with SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof; (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature
15 polypeptide sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

 The present invention also relates to methods of producing C4-dicarboxylic acids (e.g., malic acid). In one aspect, the method comprises (a) cultivating a host cell (e.g., a filamentous fungal host cell) comprising a heterologous polynucleotide encoding a C4-
20 dicarboxylic acid transporter described herein; and (b) recovering the C4-dicarboxylic acid (e.g., malic acid). In another aspect, the method comprises (a) transforming into host cell (e.g., a filamentous fungal host cell) a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein; (b) cultivating the transformed organism in a medium; and (c) recovering the C4-dicarboxylic acid (e.g., malic acid). In some aspects of
25 the methods, the host cell further comprises a heterologous polynucleotide encoding a malate dehydrogenase and/or a pyruvate carboxylase.

 The present invention also relates to a host cell (e.g., a filamentous fungal host cell, such as *Aspergillus oryzae*) comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein wherein the host cell secretes and/or is
30 capable of secreting increased levels of a C4-dicarboxylic acid (e.g., malic acid). In some aspects, the host cell further comprises a heterologous polynucleotide encoding a malate dehydrogenase and/or a pyruvate carboxylase.

 The present invention also relates to signal peptides and polynucleotides encoding the same. In one aspect, the invention relates to a polynucleotide encoding a signal peptide
35 comprising or consisting of amino acids 1 to 61 or 1 to 68 of SEQ ID NO: 2 operably linked to a gene encoding a protein. In another aspect, the invention relates to a polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO: 4

operably linked to a gene encoding a protein. In another aspect, the invention relates to a polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 68 of SEQ ID NO: 6 operably linked to a gene encoding a protein.

5 The present invention also relates to compositions comprising the polypeptides described herein, isolated polynucleotides encoding the polypeptides, nucleic acid constructs, expression vectors, recombinant host cells comprising the polynucleotides, and methods of producing the polypeptides.

Brief Description of the Figures

10 Figure 1 shows a restriction map of pShTh60.

Figure 2 shows a restriction map of pSaMF35.

Figure 3 shows the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene (*c4t737*) (SEQ ID NOs: 1 and 2, respectively).

15 Figure 4 shows a restriction map of pSaMF36.

Figure 5 shows the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene (*c4t521*) (SEQ ID NOs: 3 and 4, respectively).

20 Figure 6 shows the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus oryzae* NRRL 3488 malate dehydrogenase gene (*mdh3*) (SEQ ID NOs: 11 and 12, respectively).

Figure 7 shows a restriction map of pSaMF21.

25 Figures 8A and 8B together show the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus oryzae* NRRL 3488 pyruvate carboxylase gene (*pyc*) (SEQ ID NOs: 15 and 16, respectively).

Figure 9 shows a restriction map of pRYAN1.

Figure 10 shows a restriction map of pAaMAT737.

Figure 11 shows a restriction map of pSaMF38.

30 Figure 12 shows the genomic DNA sequence and the deduced amino acid sequence of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene (*mat737*) (SEQ ID NOs: 5 and 6, respectively).

Definitions

35 **C4-dicarboxylic acid transporter:** The term “C4-dicarboxylic acid transporter” is defined herein as a dicarboxylic acid permease that can transport malic acid, succinic acid, oxaloacetic acid, malonic acid, and/or fumaric acid outside a cell (Grobler et al., 1995, Yeast

11: 1485-1491; Camarasa et al., 2001, *Applied and Environmental Microbiology* 67: 4144-4151). A computational method to predict mitochondrially imported proteins and their targeting sequences is described by Claros and Vincens, 1996, *Eur. J. Biochem.* 241: 779-786.

5 In some aspects, the C4-dicarboxylic acid transporters have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% of the C4-dicarboxylic acid transporter activity (e.g., malic acid transporter activity) of the mature polypeptide SEQ ID NO: 2, SEQ
10 ID NO: 4, and/or SEQ ID NO: 6.

Malate dehydrogenase: The term "malate dehydrogenase" is defined herein as a malate:NAD⁺ oxidoreductase (EC 1.1.1.37) that catalyzes the reduction of oxaloacetate in the presence of NADH + H⁺ to malate and NAD⁺. For purposes of the present invention, malate dehydrogenase activity is determined according to the following procedure. The
15 assay solution consists of 1 mM oxaloacetic acid, 100 mM Tris pH 8.0, 10 mM NaHCO₃, 5 mM MgCl₂, and 0.1 mM NADH (Sigma Chemical Co., St. Louis, MO, USA). The assay solution without oxaloacetic acid as substrate is run as a control to measure background NADH degradation rates. Dilutions of 1/100, 1/500, 1/2500, and 1/12500 of each supernatant are prepared with double-distilled water. Aliquots of 270 µl of the assay solution
20 are dispensed into 96 well polystyrene flat bottom plates. A 30 µl sample of each diluted supernatant is added to initiate the assay. The reactions are monitored using a SPECTRAMAX® 340PC plate reader (Molecular Devices, Sunnyvale, CA, USA) with the following settings: 340 nm, kinetic reading. A concentration series of NADH is used to construct a standard curve and a dilution series of purified malic dehydrogenase (Sigma
25 Chemical Co., St. Louis, MO, USA) is used as a positive control. One unit of malate dehydrogenase activity equals the amount of enzyme capable of converting 1 µmole of oxaloacetate and NADH + H⁺ to malate and NAD⁺ per minute at pH 8.0, 25°C.

In some aspects, the malate dehydrogenases have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least
30 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% of the malate dehydrogenase activity of the mature polypeptide SEQ ID NO: 12.

Pyruvate carboxylase: The term "pyruvate carboxylase" is defined herein as a pyruvate:carbon-dioxide ligase (ADP-forming) (EC 6.4.1.1) that catalyzes the carboxylation
35 of pyruvate in the presence of ATP and HCO₃⁻ to oxaloacetate, ADP, and phosphate. For purposes of the present invention, pyruvate carboxylase activity is determined according to the procedure of the SIGMA® Quality Control Test procedure for pyruvate carboxylase

(Sigma Chemical Co., St. Louis, MO, USA) except the assay uses Tris buffer at pH 8.0. One unit of pyruvate carboxylase activity equals the amount of enzyme capable of converting 1 μ mole of pyruvate and CO₂ to oxaloacetate per minute at pH 7.8, 30°C.

5 In some aspects, the pyruvate carboxylases have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% of the pyruvate carboxylase activity of the mature polypeptide SEQ ID NO: 16.

Heterologous polynucleotide: The term "heterologous polynucleotide" is defined
10 herein as a polynucleotide that is not native to the host cell; a native polynucleotide in which structural modifications have been made to the coding region; a native polynucleotide whose expression is quantitatively altered as a result of a manipulation of the DNA by recombinant DNA techniques, e.g., a different (foreign) promoter; or a native polynucleotide whose expression is quantitatively altered by the introduction of one or more (e.g., two, several)
15 extra copies of the polynucleotide into the host cell.

Isolated/purified: The terms "isolated" and "purified" mean a polypeptide or polynucleotide that is removed from at least one component with which it is naturally associated. For example, a polypeptide may be at least 1% pure, e.g., at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure,
20 at least 90% pure, at least 93% pure, at least 95% pure, at least 97%, at least 98% pure, or at least 99% pure, as determined by SDS-PAGE and a polynucleotide may be at least 1% pure, e.g., at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90%, at least 93% pure, at least 95% pure, at least 97%, at least 98% pure, or at least 99% pure, as determined by agarose electrophoresis.

25 **Coding sequence:** The term "coding sequence" means a polynucleotide sequence, which specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a sequence of genomic
30 DNA, cDNA, a synthetic polynucleotide, and/or a recombinant polynucleotide.

cDNA sequence: The term "cDNA sequence" means a sequence of DNA following reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. The initial, primary RNA transcript from genomic DNA is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced
35 mRNA. A cDNA sequence lacks intervening intron sequences that may be present in the corresponding genomic DNA sequence. Accordingly, the phrase "the cDNA sequence of SEQ ID NO: X" intends the resulting sequence after the intervening intron sequences of

SEQ ID NO: X, if present, are removed. In some instances—when a referenced genomic DNA sequence lacks intervening intron sequences—a cDNA sequence may be identical to its corresponding genomic DNA sequence.

Genomic DNA sequence: The term “genomic DNA sequence” means a DNA sequence found in the genome of a source organism (e.g., a eukaryotic or prokaryotic genome). In some instances, a genomic DNA sequence from a eukaryotic genome contains one or more intervening intron sequences that are removed from the primary RNA transcript as a result of RNA splicing. Accordingly, the phrase “the genomic DNA sequence of SEQ ID NO: Y” intends the corresponding DNA sequence from the source organism which includes intervening intron sequences, if any, that are present before RNA splicing.

Mature polypeptide sequence: The term “mature polypeptide sequence” means the portion of the referenced polypeptide sequence after any post-translational sequence modifications (such as N-terminal processing and/or C-terminal truncation). In some instances, the mature polypeptide sequence may be identical to the entire referenced polypeptide sequence. In one aspect, the mature polypeptide sequence is amino acids 69 to 397 of SEQ ID NO: 2 based on the InterProScan program (The European Bioinformatics Institute) that predicts amino acids 1 to 68 of SEQ ID NO: 2 are a signal peptide. In another aspect, the mature polypeptide sequence is amino acids 62 to 397 of SEQ ID NO: 2 based on the SignalP program (Nielsen et al., 1997, *Protein Engineering* 10: 1-6) that predicts amino acids 1 to 61 of SEQ ID NO: 2 are a signal peptide. In another aspect, the mature polypeptide sequence is amino acids 18 to 418 of SEQ ID NO: 4 based on the SignalP program that predicts amino acids 1 to 17 of SEQ ID NO: 4 are a signal peptide. In another aspect, the mature polypeptide sequence is amino acids 1 to 418 of SEQ ID NO: 4 based on the InterProScan program that predicts there is no signal peptide. In another aspect, the mature polypeptide sequence is amino acids 69 to 397 of SEQ ID NO: 6 based on the InterProScan program that predicts amino acids 1 to 68 of SEQ ID NO: 6 are a signal peptide.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” means the portion of the referenced polynucleotide sequence (e.g., genomic or cDNA sequence) that encodes a mature polypeptide sequence. In some instances, the mature polypeptide coding sequence may be identical to the entire referenced polynucleotide sequence. In one aspect, the mature polypeptide coding sequence is nucleotides 205 to 1194 of SEQ ID NO: 1 based on the InterProScan program (The European Bioinformatics Institute) that predicts nucleotides 1 to 204 of SEQ ID NO: 1 encode a signal peptide. In another aspect, the mature polypeptide coding sequence is nucleotides 184 to 1194 of SEQ ID NO: 1 based on the SignalP program (Nielsen et al., 1997, *Protein Engineering* 10: 1-6) that predicts nucleotides 1 to 183 of SEQ ID NO: 1

encode a signal peptide. In another aspect, the mature polypeptide coding sequence is nucleotides 52 to 1257 of SEQ ID NO: 3 based on the SignalP program that predicts nucleotides 1 to 51 of SEQ ID NO: 3 encode a signal peptide. In another aspect, the mature polypeptide coding sequence is nucleotides 1 to 1257 of SEQ ID NO: 3 based on the InterProScan program that predicts no signal peptide. In another aspect, the mature polypeptide coding sequence is nucleotides 205 to 1194 of SEQ ID NO: 5 based on the InterProScan program that predicts nucleotides 1 to 204 of SEQ ID NO: 5 encode a signal peptide.

Fragment: The term “fragment” means a polypeptide having one or more (e.g., two, several) amino acids deleted from the amino and/or carboxyl terminus of a referenced polypeptide sequence. In one aspect, the fragment has C4-dicarboxylic acid transporter activity. In another aspect, a fragment contains at least 337 amino acid residues, e.g., at least 357 amino acid residues, or at least 377 amino acid residues of SEQ ID NO: 2. In another aspect, a fragment contains at least 355 amino acid residues, e.g., at least 375 amino acid residues or at least 395 amino acid residues of SEQ ID NO: 4. In another aspect, a fragment contains at least 337 amino acid residues, e.g., at least 357 amino acid residues or at least 377 amino acid residues of SEQ ID NO: 6.

Subsequence: The term “subsequence” means a polynucleotide having one or more (e.g., two, several) nucleotides deleted from the 5' and/or 3' end of the referenced nucleotide sequence. In one aspect, the subsequence encodes a fragment having C4-dicarboxylic acid transporter activity. In another aspect, a subsequence contains at least 1011 nucleotides, e.g., at least 1171 nucleotides, or at least 1131 nucleotides of SEQ ID NO: 1. In another aspect, a subsequence contains at least 1065 nucleotides, e.g., at least 1125 nucleotides, or at least 1185 nucleotides of SEQ ID NO: 3. In another aspect, a subsequence contains at least 1011 nucleotides, e.g., at least 1171 nucleotides or at least 1131 nucleotides of SEQ ID NO: 5.

Allelic variant: The term “allelic variant” means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the

EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle
 5 labeled "longest identity" (obtained using the *-nobrief* option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm
 10 (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained
 15 using the *-nobrief* option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Expression: The term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification,
 20 translation, post-translational modification, and secretion.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule—single-stranded or double-stranded—which is isolated from a naturally occurring gene, modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature, or synthetic, wherein the nucleic acid molecule comprises one or more (e.g.,
 25 two, several) control sequences.

Control sequence: The term "control sequence" means a nucleic acid sequence necessary for polypeptide expression. Control sequences may be native or foreign to the polynucleotide encoding the polypeptide, and native or foreign to each other. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter sequence, signal peptide sequence, and transcription terminator sequence. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.
 30

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.
 35

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences, wherein the control sequences provide for expression of the polynucleotide encoding the polypeptide. At a minimum, the expression vector comprises a promoter sequence, and transcriptional and translational stop signal sequences.

Host cell: The term “host cell” means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention (e.g., a polynucleotide encoding a C4-dicarboxylic acid transporter). The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Variant: The term “variant” means a polypeptide having activity, e.g., C4-dicarboxylic acid transporter activity, comprising an alteration, i.e., a substitution, insertion, and/or deletion of one or more (e.g., two, several) amino acid residues at one or more positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding one or more, e.g., 1-3 amino acids, adjacent to an amino acid occupying a position.

Volumetric productivity: The term “volumetric productivity” refers to the amount of referenced product produced (e.g., the amount of a C4-dicarboxylic acid produced) per volume of the system used (e.g., the total volume of media and contents therein) per unit of time.

Fermentable medium: The term “fermentable medium” refers to a medium comprising one or more (e.g., two, several) sugars, such as glucose, fructose, sucrose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides, wherein the medium is capable, in part, of being converted (fermented) by a host cell into a desired product, such as a C4-dicarboxylic acid. In some instances, the fermentation medium is derived from a natural source, such as sugar cane, starch, or cellulose, and may be the result of pretreating the source by enzymatic hydrolysis (saccharification).

Reference to “about” a value or parameter herein includes aspects that are directed to that value or parameter *per se*. For example, description referring to “about X” includes the aspect “X”.

As used herein and in the appended claims, the singular forms “a,” “or,” and “the” include plural referents unless the context clearly dictates otherwise. It is understood that the aspects of the invention described herein include “consisting” and/or “consisting essentially of” aspects.

Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

5

Detailed Description of the Invention

Polypeptides Having C4-dicarboxylic acid transporter Activity

The present invention relates to isolated polypeptides having C4-dicarboxylic acid transporter activity. In one aspect the isolated polypeptides having C4-dicarboxylic acid transporter activity are selected from:

- 10 (a) a polypeptide having at least 65% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 1, 3, or 5; the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;
- 15 (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof;
- (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; and
- 20 (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

In another aspect the isolated polypeptides having C4-dicarboxylic acid transporter activity are selected from:

- 25 (a) a polypeptide having at least 65% sequence identity to SEQ ID NO: 2, or the mature polypeptide sequence thereof;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 1, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;
- (c) a polypeptide encoded by a polynucleotide having at least 65% sequence
- 30 identity to SEQ ID NO: 1, or the mature polypeptide coding sequence thereof;
- (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, of the mature polypeptide sequence thereof; and
- (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid
- 35 transporter activity.

In another aspect the isolated polypeptides having C4-dicarboxylic acid transporter activity are selected from:

(a) a polypeptide having at least 65% sequence identity to SEQ ID NO: 4, or the mature polypeptide sequence thereof;

5 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 3, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

(c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 3, or the mature polypeptide coding sequence thereof;

10 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 4, of the mature polypeptide sequence thereof; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

15 In another aspect the isolated polypeptides having C4-dicarboxylic acid transporter activity are selected from:

(a) a polypeptide having at least 65% sequence identity to SEQ ID NO: 6, or the mature polypeptide sequence thereof;

20 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

(c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 5, or the mature polypeptide coding sequence thereof;

25 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 6, of the mature polypeptide sequence thereof; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

30 In some of these aspects, the isolated polypeptide has an amino acid sequence of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 2, or the mature polypeptide sequence thereof, which has C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide comprises an amino acid sequence that differs by no more than
35 ten amino acids, e.g., by five amino acids, by four amino acids, by three amino acids, by two amino acids, or by one amino acid from SEQ ID NO: 2, or the mature polypeptide sequence thereof.

In one aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 2, the mature polypeptide sequence of SEQ ID NO: 2, an allelic variant thereof, or a fragment of the foregoing, having C4-dicarboxylic acid transporter activity. In another aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 2.

5 In another aspect, the polypeptide comprises or consists of the mature polypeptide sequence of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises or consists of amino acids 62 to 397 or 69 to 397 of SEQ ID NO: 2.

In some of these aspects, the isolated polypeptide has an amino acid sequence of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least
10 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 4, or the mature polypeptide sequence thereof, which has C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide comprises an amino acid sequence that differs by no more than ten amino acids, e.g., by five amino acids, by four amino acids, by three amino acids, by two
15 amino acids, or by one amino acid from SEQ ID NO: 4, or the mature polypeptide sequence thereof.

In one aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 4, the mature polypeptide sequence of SEQ ID NO: 4, an allelic variant thereof, or a fragment of the foregoing, having C4-dicarboxylic acid transporter activity. In another
20 aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 4. In another aspect, the polypeptide comprises or consists of the mature polypeptide sequence of SEQ ID NO: 4. In another preferred aspect, the polypeptide comprises or consists of amino acids 52 to 418 of SEQ ID NO: 4.

In some of these aspects, the isolated polypeptide has an amino acid sequence of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least
25 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 6, or the mature polypeptide sequence thereof, which has C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide comprises an amino acid sequence that differs by no more than ten amino acids, e.g., by five amino acids, by four amino acids, by three amino acids, by two
30 amino acids, or by one amino acid from SEQ ID NO: 6, or the mature polypeptide sequence thereof.

In one aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 6, the mature polypeptide sequence of SEQ ID NO: 6, an allelic variant thereof, or a fragment of the foregoing, having C4-dicarboxylic acid transporter activity. In another
35 aspect, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 6. In another aspect, the polypeptide comprises or consists of the mature polypeptide

sequence of SEQ ID NO: 6. In another aspect, the polypeptide comprises or consists of amino acids 69 to 397 of SEQ ID NO: 6.

In one aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity are encoded by polynucleotides that hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

In another aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity are encoded by polynucleotides that hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 3, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In another aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity are encoded by polynucleotides that hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In another aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity are encoded by polynucleotides having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In one aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity is encoded by polynucleotides having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In one aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity is encoded by polynucleotides having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or

100% sequence identity to SEQ ID NO: 3, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In one aspect, the isolated polypeptides having C4-dicarboxylic acid transporter activity is encoded by polynucleotides having at least 60%, e.g., at least 65%, at least 70%,
 5 at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing.

In one aspect, the polypeptide is encoded by SEQ ID NO: 1, 3, or 5, or the mature
 10 polypeptide coding sequence thereof. In one aspect, the polypeptide is encoded by SEQ ID NO: 1 or the mature polypeptide coding sequence thereof. In one aspect, the polypeptide is encoded by SEQ ID NO: 1. In one aspect, the polypeptide is encoded by SEQ ID NO: 3 or the mature polypeptide coding sequence thereof. In one aspect, the polypeptide is encoded by SEQ ID NO: 3. In one aspect, the polypeptide is encoded by SEQ ID NO: 5 or the mature
 15 polypeptide coding sequence thereof. In one aspect, the polypeptide is encoded by SEQ ID NO: 5. In one aspect, the polypeptide is encoded by a subsequence of SEQ ID NO: 1, 3, or 5, wherein the subsequence encodes a polypeptide having C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide is encoded by a subsequence of SEQ ID NO: 1, wherein the subsequence encodes a polypeptide having C4-dicarboxylic acid transporter
 20 activity. In one aspect, the polypeptide is encoded by a subsequence of SEQ ID NO: 3, wherein the subsequence encodes a polypeptide having C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide is encoded by a subsequence of SEQ ID NO: 5, wherein the subsequence encodes a polypeptide having C4-dicarboxylic acid transporter activity.

25 In one aspect, the isolated polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof. In one aspect, the polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 2. In one aspect, the polypeptide is a variant comprising a substitution, deletion, and/or
 30 insertion of one or more amino acids of the mature polypeptide sequence of SEQ ID NO: 2. In one aspect, the polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 4. In one aspect, the polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of the mature polypeptide sequence of SEQ ID NO: 4. In one aspect, the polypeptide is a variant
 35 comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 6. In one aspect, the polypeptide is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of the mature polypeptide sequence of SEQ ID NO: 6.

Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino-terminal or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for C4-dicarboxylic acid transporter activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., 1992, *Science* 255: 306-312; Smith et al., 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver et al., 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed

by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman et al., 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire et al., 1986, *Gene* 46: 145; Ner et al., 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

In some aspects, the total number of amino acid substitutions, deletions and/or insertions of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof, is not more than 10, e.g., not more than 1, 2, 3, 4, 5, 6, 7, 8 or 9.

In another aspect, the polypeptide is a fragment of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof, wherein the fragment has C4-dicarboxylic acid transporter activity. In one aspect, the polypeptide is a fragment of SEQ ID NO: 2 or the mature polypeptide sequence thereof, wherein the fragment has C4-dicarboxylic acid transporter activity. In one aspect, the fragment contains at least 337 amino acid residues, e.g., at least 357 amino acid residues, or at least 377 amino acid residues of SEQ ID NO: 2. In another aspect, the polypeptide is a fragment of SEQ ID NO: 4 or the mature polypeptide sequence thereof, wherein the fragment has C4-dicarboxylic acid transporter activity. In one aspect, the fragment contains at least 355 amino acid residues, e.g., at least 375 amino acid residues, or at least 395 amino acid residues of SEQ ID NO: 4. In another aspect, the polypeptide is a fragment of SEQ ID NO: 6 or the mature polypeptide sequence thereof, wherein the fragment has C4-dicarboxylic acid transporter activity. In one aspect, the fragment contains at least 337 amino acid residues, e.g., at least 357 amino acid residues, or at least 377 amino acid residues of SEQ ID NO: 6.

The polypeptide may be a hybrid polypeptide in which a portion of one polypeptide is fused at the N-terminus or the C-terminus of a portion of another polypeptide.

The polypeptide may be a fused polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fused polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused

polypeptide is under control of the same promoter(s) and terminator. Fusion proteins may also be constructed using intein technology in which fusions are created post-translationally (Cooper et al., 1993, *EMBO J.* 12: 2575-2583; Dawson et al., 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin et al., 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina et al., 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson et al., 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward et al., 1995, *Biotechnology* 13: 498-503; and Contreras et al., 1991, *Biotechnology* 9: 378-381; Eaton et al., 1986, *Biochemistry* 25: 505-512; Collins-Racie et al., 1995, *Biotechnology* 13: 982-987; Carter et al., 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

Sources of Polypeptides Having C4-Dicarboxylic Acid Transporter Activity

A polypeptide having C4-dicarboxylic acid transporter activity of the present invention (e.g., a polypeptide of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof) may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

The polypeptide may be a bacterial polypeptide. For example, the polypeptide may be a gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces* polypeptide having C4-dicarboxylic acid transporter activity, or a gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, or *Ureaplasma* polypeptide.

In one aspect, the polypeptide is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* polypeptide.

In another aspect, the polypeptide is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* polypeptide.

In another aspect, the polypeptide is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* polypeptide.

The polypeptide may also be a fungal polypeptide. For example, the polypeptide may be a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*,
 5 *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*,
 10 *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* polypeptide.

In another aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*,
 15 *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide.

In another aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*,
 20 *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australeinsis*, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia setosa*, *Thielavia spededonium*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

35 In another aspect, the polypeptide is an *Aspergillus* polypeptide, e.g., an *Aspergillus aculeatus* polypeptide, such as an *Aspergillus aculeatus* polypeptide from *E. coli* NRRL B-50400, *E. coli* NRRL B-50388, or *E. coli* NRRL B-50401.

In another aspect, the polypeptide is an *Aspergillus aculeatus* polypeptide of SEQ ID NO: 2 or SEQ ID NO: 4. In another aspect, the polypeptide is an *Aspergillus aculeatus* polypeptide of SEQ ID NO: 2. In another aspect, the polypeptide is an *Aspergillus aculeatus* polypeptide of SEQ ID NO: 4.

5 It will be understood that for the aforementioned species the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

10 Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

15 The polypeptide may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The polynucleotide encoding the polypeptide may then be obtained by similarly screening a genomic or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a polypeptide has been detected with the probe(s),
20 the polynucleotide can be isolated or cloned by utilizing techniques that are well known to those of ordinary skill in the art (see, e.g., Sambrook et al., 1989, *supra*).

Polynucleotides

25 The present invention also relates to isolated polynucleotides encoding a polypeptide of the present invention (e.g., an isolated polynucleotide encoding a polypeptide of any aspect related to SEQ ID NO: 2, 4, or 6).

The techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotides from such genomic DNA can be
30 effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis et al., 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA)
35 may be used. The polynucleotides may be cloned from a strain of *Aspergillus* (e.g., *Aspergillus aculeatus*), or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleotide sequence.

The present invention also relates to an isolated polynucleotide comprising or consisting of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof, which encodes a polypeptide having C4-dicarboxylic acid transporter activity.

In one aspect, the isolated polynucleotide comprises or consists of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 1 or the mature polypeptide coding sequence thereof, which encodes a polypeptide having C4-dicarboxylic acid transporter activity.

In one aspect, the isolated polynucleotide comprises or consists of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 3 or the mature polypeptide coding sequence thereof, which encodes a polypeptide having C4-dicarboxylic acid transporter activity.

In one aspect, the isolated polynucleotide comprises or consists of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, sequence identity to SEQ ID NO: 5 or the mature polypeptide coding sequence thereof, which encodes a polypeptide having C4-dicarboxylic acid transporter activity.

Modification of a polynucleotide encoding a polypeptide of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific activity, thermostability, pH optimum, or the like. The variant may be constructed on the basis of the polynucleotide presented as the mature polypeptide coding sequence of SEQ ID NO: 1, 3, or 5, e.g., a subsequence thereof, and/or by introduction of nucleotide substitutions that do not result in a change in the amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford et al., 1991, *Protein Expression and Purification* 2: 95-107.

The present invention also relates to isolated polynucleotides encoding polypeptides of the present invention, which hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1, 3, or 5; the mature polypeptide coding sequence of SEQ ID NO: 1, 3, or 5; the full-length complementary strand thereof; or an allelic variant or subsequence of the foregoing (Sambrook et al., 1989, *supra*), as defined herein.

In one aspect, the isolated polynucleotide hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1; the mature polypeptide coding sequence of SEQ ID NO: 1; the full-length complementary strand thereof; or an allelic variant or subsequence of the foregoing (Sambrook et al., 1989, *supra*), as defined herein.

In one aspect, the isolated polynucleotide hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1; the mature polypeptide coding sequence of SEQ ID NO: 3; the full-length complementary strand thereof; or an allelic variant or subsequence of the foregoing (Sambrook et al., 1989, *supra*), as defined herein.

In one aspect, the isolated polynucleotide hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1; the mature polypeptide coding sequence of SEQ ID NO: 5; the full-length complementary strand thereof; or an allelic variant or subsequence of the foregoing (Sambrook et al., 1989, *supra*), as defined herein.

In one aspect, the polynucleotide comprises or consists of SEQ ID NO: 1, the mature polypeptide coding sequence of SEQ ID NO: 1, or the sequence contained in plasmid pAaC4T737 which is contained in *E. coli* NRRL B-50400, or a subsequence of SEQ ID NO: 1 that encodes a fragment of SEQ ID NO: 2 having C4-dicarboxylic acid transporter activity (e.g., amino acids 69-397 or 62 to 397 of SEQ ID NO: 2), such as the polynucleotide of nucleotides 205 to 1194 or 184 to 1194 of SEQ ID NO: 1.

In another aspect, the polynucleotide comprises or consists of SEQ ID NO: 3, the mature polypeptide coding sequence of SEQ ID NO: 3, or the sequence contained in plasmid pAaC4T521 which is contained in *E. coli* NRRL B-50388, or a subsequence of SEQ ID NO: 3 that encodes a fragment of SEQ ID NO: 4 having C4-dicarboxylic acid transporter activity (e.g., amino acids 18-418 of SEQ ID NO: 4), such as the polynucleotide of nucleotides 52 to 1257 of SEQ ID NO: 3.

In another aspect, the polynucleotide comprises or consists of SEQ ID NO: 5, the mature polypeptide coding sequence of SEQ ID NO: 5, or the sequence contained in plasmid pAaMAT737 which is contained in *E. coli* NRRL B-50401, or a subsequence of SEQ ID NO: 5 that encodes a fragment of SEQ ID NO: 6 having C4-dicarboxylic acid transporter activity (e.g., amino acids 69-397 of SEQ ID NO: 6), such as the polynucleotide of nucleotides 205 to 1194 of SEQ ID NO: 5.

The polynucleotide of SEQ ID NO: 1, 3, or 5, or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 2, 4, or 6, or a fragment thereof; may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having C4-dicarboxylic acid transporter activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having C4-dicarboxylic acid transporter activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, 3, or 5, or a subsequence thereof, the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding SEQ ID NO: 1, 3, or 5; the mature polypeptide coding sequence of SEQ ID NO: 1, 3, or 5; a full-length complementary strand thereof; or a subsequence of the foregoing; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

In one aspect, the nucleic acid probe is SEQ ID NO: 1, 3, or 5. In one aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1, 3, or 5. In

another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2, 4, or 6, or a fragment thereof. In another aspect, the nucleic acid probe is SEQ ID NO: 1. In another aspect, the nucleic acid probe is SEQ ID NO: 3. In another aspect, the nucleic acid probe is SEQ ID NO: 5. In another aspect, the nucleic acid probe is the polynucleotide contained in plasmid pAaC4T737 which is contained in *E. coli* NRRL B-50400, wherein the polynucleotide encodes a polypeptide having C4-dicarboxylic acid transporter activity. In another aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAaC4T737 which is contained in *E. coli* NRRL B-50400, wherein the polypeptide has C4-dicarboxylic acid transporter activity. In another aspect, the nucleic acid probe is the polynucleotide contained in plasmid pAaC4T521 which is contained in *E. coli* NRRL B-50388, wherein the polynucleotide encodes a polypeptide having C4-dicarboxylic acid transporter activity. In another aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAaC4T521 which is contained in *E. coli* NRRL B-50388, wherein the polypeptide has C4-dicarboxylic acid transporter activity. In another aspect, the nucleic acid probe is the polynucleotide contained in plasmid pAaMAT737 which is contained in *E. coli* NRRL B-50401, wherein the polynucleotide encodes a polypeptide having C4-dicarboxylic acid transporter activity. In another aspect, the nucleic acid probe is the mature polypeptide coding sequence contained in plasmid pAaMAT737 which is contained in *E. coli* NRRL B-50401, wherein the polypeptide has C4-dicarboxylic acid transporter activity.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), at 50°C (low stringency), at 55°C (medium stringency), at 60°C (medium-high stringency), at 65°C (high stringency), and at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once

in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

Nucleic Acid Constructs

5 The present invention also relates to nucleic acid constructs comprising a polynucleotide of the present invention operably linked to one or more (e.g., two, several) control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences. The present invention also embraces recombinant host cells and methods utilizing nucleic acid constructs comprising a
10 heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (and/or a malate dehydrogenase, or a pyruvate carboxylase described herein) linked to one or more control sequences that direct expression in a suitable host cell under conditions compatible with the control sequence(s). Such nucleic acid constructs may be used in any of the host cells and methods describe herein.

15 The polynucleotides described herein may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

20 The control sequence may be a promoter sequence, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter sequence contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the host cell of choice including mutant, truncated, and
25 hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Each polynucleotide described herein may be operably linked to a promoter that is foreign to the polynucleotide. For example, in one aspect, the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter is operably linked to a promoter that is foreign to
30 the polynucleotide. In another aspect, the heterologous polynucleotide encoding a malate dehydrogenase is operably linked to promoter foreign to the polynucleotide. In another aspect, the heterologous polynucleotide encoding a pyruvate carboxylase is operably linked to promoter foreign to the polynucleotide.

Examples of suitable promoters for directing the transcription of the nucleic acid
35 constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus*

stearothermophilus maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *E. coli* *lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer et al., 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert et al., 1980, *Scientific American*, 242: 74-94; and in Sambrook et al., 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from a gene encoding a neutral alpha-amylase in *Aspergilli* in which the untranslated leader has been replaced by an untranslated leader from a gene encoding triose phosphate isomerase in *Aspergilli*; non-limiting examples include modified promoters from the gene encoding neutral alpha-amylase in *Aspergillus niger* in which the untranslated leader has been replaced by an untranslated leader from the gene encoding triose phosphate isomerase in *Aspergillus nidulans* or *Aspergillus oryzae*); and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos et al., 1992, *Yeast* 8: 423-488.

The control sequence may also be a suitable transcription terminator sequence, which is recognized by a host cell to terminate transcription. The terminator sequence is

operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any terminator that is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, *supra*.

The control sequence may also be a suitable leader sequence, when transcribed is a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell of choice may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be

required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into
5 the secretory pathway of a host cell of choice may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*,
10 *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and
15 *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos et al., 1992, *supra*.

20 The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may
25 be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present at the N-terminus of a polypeptide, the propeptide sequence is positioned next to the N-terminus of a
30 polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response
35 to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus*

niger glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The present invention also embraces recombinant host cells and methods utilizing recombinant expression vectors comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter (and/or a malate dehydrogenase or a pyruvate carboxylase); as well as a promoter; and transcriptional and translational stop signals. Such recombinant expression vectors may be used in any of the host cells and methods described herein.

The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more (e.g., two, several) convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

In one aspect, each polynucleotide encoding a C4-dicarboxylic acid transporter, a malate dehydrogenase, and/or a pyruvate carboxylase described herein is contained on an independent vector. In one aspect, at least two of the polynucleotides are contained on a single vector. In one aspect, all the polynucleotides encoding the C4-dicarboxylic acid transporter, the malate dehydrogenase, and the pyruvate carboxylase are contained on a single vector.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication,

e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated.

5 Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more (e.g., two, several) selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A
10 selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells
15 are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents
20 thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

25 For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the
30 likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target
35 sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems et al., 1991, *Gene* 98: 61-67; Cullen et al., 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, *supra*).

Malate Dehydrogenases and Polynucleotides Encoding Malate Dehydrogenases

In some aspects of the recombinant host cells and methods of use thereof, the host cells have malate dehydrogenase activity. In some aspects, the host cells comprise a heterologous polynucleotide encoding a malate dehydrogenase. The malate dehydrogenase can be any malate dehydrogenase that is suitable for practicing the invention. In one aspect, the malate dehydrogenase is an enzyme that is present in the cytosol of the host cell.

In one aspect of the recombinant host cells and methods described herein, the malate dehydrogenase is (a) a malate dehydrogenase having at least 60% sequence identity to SEQ ID NO: 12 or the mature polypeptide sequence thereof; (b) a malate dehydrogenase encoded by a polynucleotide that hybridizes under low stringency conditions with (i) SEQ ID

NO: 11 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii); (c) a malate dehydrogenase encoded by a polynucleotide having at least 60% sequence identity to (iv) SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 11 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v); (d) a malate dehydrogenase variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 12 or the mature polypeptide sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has malate dehydrogenase activity.

In one aspect, the malate dehydrogenase comprises or consists of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 12 or the mature polypeptide sequence thereof. In one aspect, the malate dehydrogenase comprises an amino acid sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 12 or the mature polypeptide sequence thereof.

In one aspect, the malate dehydrogenase comprises or consists of the amino acid sequence of SEQ ID NO: 12, the mature polypeptide sequence of SEQ ID NO: 12, an allelic variant thereof, or a fragment of the foregoing, having malate dehydrogenase activity. In another aspect, the malate dehydrogenase comprises or consists of the amino acid sequence of SEQ ID NO: 12. In another aspect, the malate dehydrogenase comprises or consists of the mature polypeptide sequence of SEQ ID NO: 12. In another aspect, the malate dehydrogenase comprises or consists of amino acids 1 to 330 of SEQ ID NO: 12.

In one aspect, the malate dehydrogenase is encoded by a polynucleotide that hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *supra*).

In one aspect, the malate dehydrogenase is encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to (iv) SEQ ID NO: 11 or

the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 11 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v).

5 In one aspect, the malate dehydrogenase is encoded by SEQ ID NO: 11, or the mature polypeptide coding sequence thereof. In one aspect, the malate dehydrogenase is encoded by SEQ ID NO: 11. In one aspect, the malate dehydrogenase is encoded by the mature polypeptide coding sequence of SEQ ID NO: 11. In one aspect, the malate dehydrogenase is encoded by a subsequence of SEQ ID NO: 11, wherein the subsequence encodes a polypeptide having malate dehydrogenase activity. In one aspect, the
10 subsequence contains at least 885 nucleotides, e.g., at least 930 nucleotides or at least 975 nucleotides of SEQ ID NO: 11.

In one aspect, the malate dehydrogenase is a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 12, or the mature polypeptide sequence thereof, as described *supra*. In one aspect, the malate
15 dehydrogenase is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 12. In one aspect, the malate dehydrogenase is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of the mature polypeptide sequence of SEQ ID NO: 12. In some aspects, the total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide sequence of
20 SEQ ID NO: 12 or the mature polypeptide sequence thereof is not more than 10, e.g., not more than 1, 2, 3, 4, 5, 6, 7, 8 or 9.

In another aspect, the malate dehydrogenase is a fragment of SEQ ID NO: 12, or the mature polypeptide sequence thereof, wherein the fragment has malate dehydrogenase activity. In one aspect, the fragment contains at least 295 amino acid residues, e.g., at least
25 310 amino acid residues, or at least 325 amino acid residues of SEQ ID NO: 12.

The malate dehydrogenase may also be an allelic variant or artificial variant of a malate dehydrogenase.

The malate dehydrogenase can also include fused polypeptides or cleavable fusion polypeptides, as described *supra*.

30 Techniques used to isolate or clone a polynucleotide encoding a malate dehydrogenase are described *supra*.

The polynucleotide of SEQ ID NO: 11; or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 12; or a fragment thereof; may be used to design nucleic acid probes to identify and clone DNA encoding malate dehydrogenases from strains
35 of different genera or species, as described *supra*. Such probes are encompassed by the present invention. A genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a malate

dehydrogenase, as described *supra*.

In one aspect, the nucleic acid probe is SEQ ID NO: 11. In another aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 11. In another aspect, the nucleic acid probe is a polynucleotide sequence that encodes SEQ ID NO: 12, the mature polypeptide sequence thereof, or a fragment of the foregoing.

For long probes of at least 100 nucleotides in length, very low to very high stringency and washing conditions are defined as described *supra*. For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency and washing conditions are defined as described *supra*.

The malate dehydrogenase may be obtained from microorganisms of any genus. In one aspect, the malate dehydrogenase may be a bacterial, a yeast, or a filamentous fungal malate dehydrogenase obtained from the microorganisms described herein. In another aspect, the malate dehydrogenase is an *Aspergillus oryzae* malate dehydrogenase, e.g., the *Aspergillus oryzae* malate dehydrogenase of SEQ ID NO: 12.

Other malate dehydrogenases that can be used to practice the present invention include, but are not limited to, a *Aspergillus nidulans* malate dehydrogenase (AN6717.1; SIMS et al., 2004, *Mycol. Res.* 108 : 853–857); *Aspergillus niger* malate dehydrogenase (An16g00120; Pel et al., 2007, *Nature Biotechnology* 25: 221-231); *Phytophthora infestans* malate dehydrogenase (PITG 13614.1; Calcagno et al., 2009, *Mycological Research* 113: 771–781); *Saccharomyces cerevisiae* malate dehydrogenase (YKL085W; McAlister-Henn and Thompson, 1987, *J. Bacteriol.* 169: 5157–5166); *Talaromyces emersonii* malate dehydrogenase (AF439996, AF487682; Maloney et al., 2004, *Eur. J. Biochem.* 271: 3115–3126); and *Ustilago maydis* malate dehydrogenase (um00403, um11161; McCann and Snetselaar, 2008, *Fungal Genetics and Biology* 45: S77–S87), the *Aspergillus oryzae* malate dehydrogenase of SEQ ID NO: 20 (encoded by the polynucleotide sequence of SEQ ID NO: 19; see U.S. Application No. 12/870,523, entitled “Methods for Improving Malic Acid Production in Filamentous Fungi” filed August 27, 2010), or any aspect of the malate dehydrogenase described in the respective reference therein.

The invention embraces any aspect of sequence identity, hybridization, variants and fragments described herein as applied to the malate dehydrogenase polypeptide sequences and polynucleotide sequences described above. For example, in one aspect, the malate dehydrogenase is (a) a malate dehydrogenase having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 20, or the mature polypeptide sequence thereof; (b) a malate dehydrogenase encoded by a polynucleotide that hybridizes under low stringency conditions, e.g., medium stringency conditions, medium-high

stringency conditions, high stringency conditions, or very high stringency conditions with (i) SEQ ID NO: 19 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 19 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of the (i) or (ii); (c) a malate dehydrogenase encoded by a polynucleotide having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to (iv) SEQ ID NO: 19 or the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 19 or the mature polypeptide coding sequence thereof, or (vi) the full-length complementary strand of the (iv) or (v); (d) a malate dehydrogenase variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 20 or the mature polypeptide sequence thereof; or (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has malate dehydrogenase activity.

The malate dehydrogenase may also be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) as described *supra*.

Pyruvate Carboxylases and Polynucleotides Encoding Pyruvate Carboxylases

In some aspects of the recombinant host cells and methods of use thereof, the host cells have pyruvate carboxylase activity. In some aspects, the host cells comprise a heterologous polynucleotide encoding a pyruvate carboxylase. The pyruvate carboxylase can be any pyruvate carboxylase that is suitable for practicing the invention. In one aspect, the pyruvate carboxylase is an enzyme that is present in the cytosol of the host cell.

In one aspect of the recombinant host cells and methods described herein, the pyruvate carboxylase is (a) a pyruvate carboxylase having at least 60% sequence identity to SEQ ID NO: 16 or the mature polypeptide sequence thereof; (b) a pyruvate carboxylase encoded by a polynucleotide that hybridizes under low stringency conditions with (i) SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii); (c) a pyruvate carboxylase encoded by a polynucleotide having at least 60% sequence identity to (iv) SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v); (d) a pyruvate carboxylase variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 16 or the mature polypeptide

sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has pyruvate carboxylase activity.

In one aspect, the pyruvate carboxylase comprises or consists of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%,
5 at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 16, or the mature polypeptide sequence thereof. In one aspect, the pyruvate carboxylase comprises an amino acid sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more
10 than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 16 or the mature polypeptide sequence thereof.

In one aspect, the pyruvate carboxylase comprises or consists of the amino acid sequence of SEQ ID NO: 16, the mature polypeptide sequence of SEQ ID NO: 16, an allelic variant thereof, or a fragment of the foregoing, having pyruvate carboxylase activity. In
15 another aspect, the pyruvate carboxylase comprises or consists of the amino acid sequence of SEQ ID NO: 16. In another aspect, the pyruvate carboxylase comprises or consists of the mature polypeptide sequence of SEQ ID NO: 16. In another aspect, the pyruvate carboxylase comprises or consists of amino acids 1 to 1193 of SEQ ID NO: 16.

In one aspect, the pyruvate carboxylase is encoded by a polynucleotide that
20 hybridizes under at least low stringency conditions, e.g., medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T.
25 Maniatis, 1989, *supra*).

In one aspect, the pyruvate carboxylase is encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to (iv) SEQ ID NO: 15 or
30 the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v).

In one aspect, the pyruvate carboxylase is encoded by SEQ ID NO: 15 or the mature polypeptide coding sequence thereof. In one aspect, the pyruvate carboxylase is encoded by
35 SEQ ID NO: 15. In one aspect, the pyruvate carboxylase is encoded by the mature polypeptide coding sequence of SEQ ID NO: 15. In one aspect, the pyruvate carboxylase is encoded by a subsequence of SEQ ID NO: 15, wherein the subsequence encodes a

polypeptide having pyruvate carboxylase activity. In one aspect, the subsequence contains at least 3060 nucleotides, e.g., at least 3240 nucleotides or at least 3420 nucleotides of SEQ ID NO: 15.

5 In one aspect, the pyruvate carboxylase is a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 16, or the mature polypeptide sequence thereof, as described *supra*. In one aspect, the pyruvate carboxylase is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of SEQ ID NO: 16. In one aspect, the pyruvate carboxylase is a variant comprising a substitution, deletion, and/or insertion of one or more amino acids of the
10 mature polypeptide sequence of SEQ ID NO: 16. In some aspects, the total number of amino acid substitutions, deletions and/or insertions of SEQ ID NO: 16 or the mature polypeptide sequence thereof is not more than 16, e.g., not more than 1, 2, 3, 4, 5, 6, 7, 8 or 9.

15 In another aspect, the pyruvate carboxylase is a fragment of SEQ ID NO: 16, or the mature polypeptide sequence thereof, wherein the fragment has pyruvate carboxylase activity. In one aspect, the fragment contains at least 1020 amino acid residues, e.g., at least 1080 amino acid residues, or at least 1140 amino acid residues of SEQ ID NO: 16.

The pyruvate carboxylase may also be an allelic variant or artificial variant of a pyruvate carboxylase.

20 The pyruvate carboxylase can also include fused polypeptides or cleavable fusion polypeptides, as described *supra*.

The pyruvate carboxylase can also be a variant of a mitochondrial pyruvate carboxylase, such that *in vivo* importation into the mitochondria is reduced thereby increasing the level of the pyruvate carboxylase variant in the cytosol.

25 Techniques used to isolate or clone a polynucleotide encoding a pyruvate carboxylase are described *supra*.

The polynucleotide of SEQ ID NO: 15 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO: 16 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding pyruvate carboxylases from strains
30 of different genera or species, as described *supra*. Such probes are encompassed by the present invention. A genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a pyruvate carboxylase, as described *supra*.

35 In one aspect, the nucleic acid probe is SEQ ID NO: 15. In another aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 15. In another aspect, the nucleic acid probe is a polynucleotide sequence that encodes SEQ ID NO: 16, the mature polypeptide sequence thereof, or a fragment of the foregoing.

For long probes of at least 100 nucleotides in length, very low to very high stringency and washing conditions are defined as described *supra*. For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency and washing conditions are defined as described *supra*.

5 The pyruvate carboxylase may be obtained from microorganisms of any genus. In one aspect, the pyruvate carboxylase may be a bacterial, a yeast, or a filamentous fungal pyruvate carboxylase obtained from the microorganisms described herein. In another aspect, the pyruvate carboxylase is an *Aspergillus oryzae* pyruvate carboxylase, e.g., the *Aspergillus oryzae* pyruvate carboxylase of SEQ ID NO: 16.

10 Other pyruvate carboxylases that can be used to practice the present invention include, but are not limited to, a *Aspergillus clavatus* NRRL 1 pyruvate carboxylase (XP_001271664; Direct Submission, Submitted (26-OCT-2006), The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA); *Aspergillus fumigatus* Af293 pyruvate carboxylase (XP_752054; Nierman et al., 2005, *Nature* 438: 1151-1156);
 15 *Aspergillus nidulans* FGSC A4 pyruvate carboxylase (XP_662066; Galagan et al., 2005, *Nature* 438: 1105-1115); *Aspergillus niger* pyruvate carboxylase (An15g02820; Pel et al., 2007, *Nature Biotechnology* 25: 221 – 231; ASPNG 5061; Panneman et al., Submitted (JUL-1998) to the EMBL/GenBank/DDBJ databases); *Aspergillus terreus* pyruvate carboxylase (O93918; Direct Submission, Submitted (OCT-1998) The Institute for Genomic
 20 Research, 9712 Medical Center Drive, Rockville, MD 20850, USA); *Magnaporthe grisea* 70-15 pyruvate carboxylase (XP_367852; Direct Submission, Submitted (26-SEP-2005) Broad Institute of MIT and Harvard, 320 Charles Street, Cambridge, MA 02142, USA); *Neurospora crassa* OR74A pyruvate carboxylase (XP_965636; Galagan et al., 2003, *Nature* 422: 859-868); *Rhizopus oryzae* pyruvate carboxylase (RO3G_06931.1); *Saccharomyces cerevisiae*
 25 pyruvate carboxylase (NP_009777; Gaffeau et al., 1996, *Science* 274: 546-547); *Schizosaccharomyces pombe* pyruvate carboxylase (NP_595900; Direct Submission, Submitted (29-JUN-2007) European Schizosaccharomyces genome sequencing project, Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA); and *Ustilago maydis* pyruvate carboxylase (um01054; McCann and Snetselaar, 2008,
 30 *Fungal Genetics and Biology* 45: S77–S87). The invention embraces any aspect of sequence identity, hybridization, variants and fragments described herein as applied to the malate dehydrogenase polypeptide sequences and polynucleotide sequences described above.

 The pyruvate carboxylase may also be identified and obtained from other sources
 35 including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) as described *supra*.

Host Cells

The present invention also relates to recombinant host cells comprising a polynucleotide described herein operably linked to one or more (e.g., two, several) control sequences that direct the production of a polypeptides described herein. The invention also embraces recombinant host cells comprising one or more polynucleotide(s) described herein which may be operably linked to one or more control sequences that direct the expression of one or more of the described polypeptides for the recombinant production of a C4-dicarboxylic acid, as well as methods of using such host cells for the production of a C4-dicarboxylic acid. The host cell may comprise any one or combination of a plurality of the polynucleotides described. For example, in one aspect, the recombinant host cell comprises a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter; and optionally comprises a heterologous polynucleotide encoding a heterologous polynucleotide encoding a malate dehydrogenase, and/or a heterologous polynucleotide encoding pyruvate decarboxylase; wherein the host cell produces (or is capable of producing) a greater amount of a C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide encoding the C4-dicarboxylic acid transporter when cultivated under the same conditions.

In one aspect, the recombinant host cell comprises:

(1) a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter, such as a C4-dicarboxylic acid transporter selected from: (a) a C4-dicarboxylic acid transporter having at least 60% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; (b) a C4-dicarboxylic acid transporter encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (c) a C4-dicarboxylic acid transporter encoded by a polynucleotide having at least 60% sequence identity to SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (d) a C4-dicarboxylic acid transporter variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity;

(2) an optional heterologous second polynucleotide encoding a malate dehydrogenase, such as a malate dehydrogenase selected from: (a) a malate dehydrogenase having at least 60% sequence identity to SEQ ID NO: 12 or the mature polypeptide sequence thereof; (b) a malate dehydrogenase encoded by a polynucleotide that hybridizes under low stringency conditions with (i) SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 11 or the

mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii); (c) a malate dehydrogenase encoded by a polynucleotide having at least 60% sequence identity to (iv) SEQ ID NO: 11 or the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 11 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v); (d) a malate dehydrogenase variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 12 or the mature polypeptide sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has malate dehydrogenase activity; and

(3) an optional heterologous third polynucleotide encoding a pyruvate carboxylase, such as a pyruvate carboxylase selected from: (a) a pyruvate carboxylase having at least 60% sequence identity to SEQ ID NO: 16 or the mature polypeptide sequence thereof; (b) a pyruvate carboxylase encoded by a polynucleotide that hybridizes under low stringency conditions with (i) SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, (ii) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, or (iii) the full-length complementary strand of (i) or (ii); (c) a pyruvate carboxylase encoded by a polynucleotide having at least 60% sequence identity to (iv) SEQ ID NO: 15 or the mature polypeptide coding sequence thereof, (v) the cDNA sequence of SEQ ID NO: 15 or the mature polypeptide coding sequence thereof; or (vi) the full-length complementary strand of (iv) or (v); (d) a pyruvate carboxylase variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 16 or the mature polypeptide sequence thereof; and (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has pyruvate carboxylase activity;

wherein the host cell produces (or is capable of producing) a greater amount of a C4-dicarboxylic acid (e.g., malic acid) compared to the host cell without the one or more polynucleotide(s) (e.g., without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter), when cultivated under the same conditions.

In one aspect, the host cell comprises a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (e.g., SEQ ID NO: 1, 3, 5, or any described aspect thereof) and a heterologous polynucleotide encoding a malate dehydrogenase. In the present invention, the malate dehydrogenase can be any malate dehydrogenase that is suitable for practicing the present invention, as described *supra*. In another aspect, the host cell comprises a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (e.g., SEQ ID NO: 1, 3, 5, or any described aspect thereof) and a heterologous polynucleotide encoding a pyruvate carboxylase. In the present invention, the pyruvate carboxylase can be any pyruvate carboxylase that is suitable for practicing the present invention, as described *supra*. In particular, the pyruvate carboxylase is preferably

an enzyme that is present in the cytosol of the host cell. In one aspect, the host cell comprises a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (e.g., SEQ ID NO: 1, 3, 5, or any described aspect thereof), a second heterologous polynucleotide encoding a malate dehydrogenase, and a third heterologous polynucleotide encoding a pyruvate carboxylase.

A construct or vector (or multiple constructs or vectors) comprising the one or more (e.g., two, several) polynucleotide(s) is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The aspects described below apply to the host cells, *per se*, as well as methods using the host cells.

The host cell may be any cell capable of the recombinant production of a polypeptide of the present invention, e.g., a prokaryote or a eukaryote, and/or any cell (e.g., any filamentous fungal cell) capable of the recombinant production of a C4-dicarboxylic acid (e.g., malic acid).

The prokaryotic host cell may be any gram-positive or gram-negative bacterium. Gram-positive bacteria include, but not limited to, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. Gram-negative bacteria include, but not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

The bacterial host cell may also be any *Streptomyces* cell including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The introduction of DNA into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-

829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower et al., 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may, for instance, be effected by protoplast transformation and electroporation (see, e.g., Gong et al., 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, e.g., Mazodier et al., 1989, *J. Bacteriol.* 171: 3583-3585), or by transduction (see, e.g., Burke et al., 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may, for instance, be effected by electroporation (see, e.g., Choi et al., 2006, *J. Microbiol. Methods* 64: 391-397) or by conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), by protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207, by electroporation (see, e.g., Buckley et al., 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or by conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth et al., *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth et al., 1995, *supra*).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth et al., 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

In one aspect, the host cell is an *Aspergillus* host cell. In another aspect, the host cell is *Aspergillus oryzae*.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023 and Yelton et al., 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier et al., 1989,

Gene 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *J. Bacteriol.* 153: 163; and Hinnen et al., 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

In some aspects, the host cell comprises one or more (e.g., two, several) polynucleotide(s) described herein, wherein the host cell secretes (and/or is capable of secreting) an increased level of C4-dicarboxylic acid compared to the host cell without the one or more polynucleotide(s) when cultivated under the same conditions. In some aspects, the host cell secretes and/or is capable of secreting an increased level of C4-dicarboxylic acid (e.g., malic acid) of at least 5%, e.g., at least 10%, at least 15%, at least 20%, at least 25%, at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cell without the one or more polynucleotide(s) (e.g., without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter), when cultivated under the same conditions.

In any of the aspects of the recombinant host cells and methods described herein, the C4-dicarboxylic acid may be malic acid, succinic acid, oxaloacetic acid, malonic acid, or fumaric acid, or combinations thereof. In some aspects, the C4-dicarboxylic acid is malic acid, succinic acid, or fumaric acid, or combinations thereof. In some aspects, the C4-dicarboxylic acid is malic acid or fumaric acid, or a combination of malic acid and fumaric acid. In some aspects, the C4-dicarboxylic acid is malic acid.

In any of these aspects, the host cell produces (and/or is capable of producing) a C4-dicarboxylic acid at a yield of at least than 10%, e.g., at least than 20%, at least than 30%, at least than 40%, at least than 50%, at least than 60%, at least than 70%, at least than 80%, or at least than 90%, of theoretical.

In any of these aspects, the recombinant host has an C4-dicarboxylic acid volumetric productivity (e.g., malic acid volumetric productivity) greater than about 0.1 g/L per hour, e.g., greater than about 0.2 g/L per hour, 0.5 g/L per hour, 0.6 g/L per hour, 0.7 g/L per hour, 0.8 g/L per hour, 0.9 g/L per hour, 1.0 g/L per hour, 1.1 g/L per hour, 1.2 g/L per hour, 1.3 g/L per hour, 1.5 g/L per hour, 1.75 g/L per hour, 2.0 g/L per hour, 2.25 g/L per hour, 2.5 g/L per hour, or 3.0 g/L per hour; or between about 0.1 g/L per hour and about 2.0 g/L per hour, e.g., between about 0.3 g/L per hour and about 1.7 g/L per hour, about 0.5 g/L per hour and about 1.5 g/L per hour, about 0.7 g/L per hour and about 1.3 g/L per hour, about 0.8 g/L per hour and about 1.2 g/L per hour, or about 0.9 g/L per hour and about 1.1 g/L per hour.

The recombinant host cells may be cultivated in a nutrient medium suitable for production of the C4-dicarboxylic acid transporter, malate dehydrogenase, or pyruvate carboxylase using methods well known in the art, as described below.

The C4-dicarboxylic acid transporter, malate dehydrogenase, and pyruvate carboxylase, and activities thereof, can be detected using methods known in the art. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. See, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999); and Hanai et al., *Appl. Environ. Microbiol.* 73:7814-7818 (2007)).

Methods of Production

The present invention also relates to methods of producing a polypeptide described herein (e.g., a polypeptide comprising or consisting of SEQ ID NO: 2, 4, 6, or any described aspect thereof), comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In one aspect, the cell is of the genus *Aspergillus*. In another aspect, the cell is *Aspergillus aculeatus*. In a another aspect, the cell is *E. coli* NRRL B-50400, *E. coli* NRRL B-50388, or *E. coli* NRRL B-50401.

The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a recombinant host cell of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

The host cells are cultivated in a nutrient medium suitable for production of the C4-dicarboxylic acid transporter using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The C4-dicarboxylic acid transporter may be detected using methods known in the art that are specific for the polypeptides, as described *supra*. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

5 The polypeptide may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, 10 VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing a polypeptide is used as a source of the polypeptide.

Plants

15 The present invention also relates to plants, e.g., a transgenic plant, plant part, or plant cell, comprising an isolated polynucleotide of the present invention so as to express and produce the polypeptide in recoverable quantities. The polypeptide may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the polypeptide may be used as such for improving the quality of a food or feed, e.g., improving nutritional 20 value, palatability, and rheological properties, or to destroy an antinutritive factor.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

25 Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family Brassicaceae), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, 30 parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, e.g., embryos, 35 endosperms, aleurone and seeds coats.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

The transgenic plant or plant cell expressing a polypeptide may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by incorporating one or more (e.g., two, several) expression constructs encoding a polypeptide into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

The expression construct is conveniently a nucleic acid construct that comprises a polynucleotide encoding a polypeptide operably linked with appropriate regulatory sequences required for expression of the polynucleotide in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences, is determined, for example, on the basis of when, where, and how the polypeptide is desired to be expressed. For instance, the expression of the gene encoding a polypeptide may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are, for example, described by Tague et al., 1988, *Plant Physiology* 86: 506.

For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, and the rice actin 1 promoter may be used (Franck et al., 1980, *Cell* 21: 285-294; Christensen et al., 1992, *Plant Mol. Biol.* 18: 675-689; Zhang et al., 1991, *Plant Cell* 3: 1155-1165). Organ-specific promoters may be, for example, a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards and Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu et al., 1998, *Plant Cell Physiol.* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad et al., 1998, *J. Plant Physiol.* 152: 708-711), a promoter from a seed oil body protein (Chen et al., 1998, *Plant Cell Physiol.* 39: 935-941), the storage protein *napA* promoter from *Brassica napus*, or any other seed specific promoter known in the art, e.g., as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcS* promoter from rice or tomato (Kyoizuka et al., 1993, *Plant Physiol.* 102: 991-1000), the chlorella virus adenine methyltransferase gene promoter (Mittra and Higgins, 1994, *Plant Mol. Biol.* 26: 85-93), the *aldP* gene promoter from rice (Kagaya et al., 1995, *Mol. Gen. Genet.* 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu et al., 1993, *Plant Mol. Biol.* 22: 573-588). Likewise, the promoter may inducible by abiotic treatments such as

temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, e.g., ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

5 A promoter enhancer element may also be used to achieve higher expression of a polypeptide in the plant. For instance, the promoter enhancer element may be an intron that is placed between the promoter and the polynucleotide encoding a polypeptide. For instance, Xu et al., 1993, *supra*, disclose the use of the first intron of the rice actin 1 gene to enhance expression.

10 The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation, and electroporation (Gasser et al., 1990, *Science* 244: 1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto et al., 1989, *Nature* 338: 274).

20 Presently, *Agrobacterium tumefaciens*-mediated gene transfer is the method of choice for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Mol. Biol.* 19: 15-38) and can also be used for transforming monocots, although other transformation methods are often used for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant J.* 2: 275-281; Shimamoto, 1994, *Curr. Opin. Biotechnol.* 5: 158-162; Vasil et al., 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh et al., 1993, *Plant Mol. Biol.* 21: 415-428. Additional transformation methods for use in accordance with the present disclosure include those described in U.S. Patent Nos. 6,395,966 and 7,151,204.

30 Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using, for example, co-transformation with two separate T-DNA constructs or site specific excision of the selection gene by a specific recombinase.

35 In addition to direct transformation of a particular plant genotype with a construct prepared according to the present invention, transgenic plants may be made by crossing a plant having the construct to a second plant lacking the construct. For example, a construct encoding a polypeptide can be introduced into a particular plant variety by crossing, without

the need for ever directly transforming a plant of that given variety. Therefore, the present invention encompasses not only a plant directly regenerated from cells which have been transformed in accordance with the present invention, but also the progeny of such plants. As used herein, progeny may refer to the offspring of any generation of a parent plant prepared in accordance with the present invention. Such progeny may include a DNA construct prepared in accordance with the present invention, or a portion of a DNA construct prepared in accordance with the present invention. Crossing results in the introduction of a transgene into a plant line by cross pollinating a starting line with a donor plant line. Non-limiting examples of such steps are further articulated in U.S. Patent No: 7,151,204.

Plants may be generated through a process of backcross conversion. For example, plants include plants referred to as a backcross converted genotype, line, inbred, or hybrid.

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

The present invention also relates to methods of producing a polypeptide of the present invention comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

Removal or Reduction of C4-Dicarboxylic Acid Transporter Activity

The present invention also relates to methods of producing a mutant of a parent cell, which comprises disrupting or deleting a polynucleotide, or a portion thereof, encoding a polypeptide of the present invention, which results in the mutant cell producing less of the polypeptide than the parent cell when cultivated under the same conditions.

The mutant cell may be constructed by reducing or eliminating expression of the polynucleotide using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. In a preferred aspect, the polynucleotide is inactivated. The polynucleotide to be modified or inactivated may be, for example, the coding region or a part thereof essential for activity, or a regulatory element required for the expression of the coding region. An example of such a regulatory or control sequence may be a promoter

sequence or a functional part thereof, i.e., a part that is sufficient for affecting expression of the polynucleotide. Other control sequences for possible modification include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, signal peptide sequence, transcription terminator, and transcriptional activator.

5 Modification or inactivation of the polynucleotide may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the polynucleotide has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA
10 sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

 Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane
15 sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

 When such agents are used, the mutagenesis is typically performed by incubating the parent cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and screening and/or selecting for mutant cells exhibiting reduced or no expression of the gene.

20 Modification or inactivation of the polynucleotide may be accomplished by introduction, substitution, or removal of one or more (e.g., two, several) nucleotides in the gene or a regulatory element required for the transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change in the open reading frame. Such
25 modification or inactivation may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. Although, in principle, the modification may be performed *in vivo*, i.e., directly on the cell expressing the polynucleotide to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

30 An example of a convenient way to eliminate or reduce expression of a polynucleotide is based on techniques of gene replacement, gene deletion, or gene disruption. For example, in the gene disruption method, a nucleic acid sequence corresponding to the endogenous polynucleotide is mutagenized *in vitro* to produce a defective nucleic acid sequence that is then transformed into the parent cell to produce a
35 defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous polynucleotide. It may be desirable that the defective polynucleotide also encodes a marker that may be used for selection of transformants in which the

polynucleotide has been modified or destroyed. In a particularly preferred aspect, the polynucleotide is disrupted with a selectable marker such as those described herein.

5 The present invention also relates to methods of inhibiting the expression of a polypeptide having C4-dicarboxylic acid transporter activity in a cell, comprising administering to the cell or expressing in the cell a double-stranded RNA (dsRNA) molecule, wherein the dsRNA comprises a subsequence of a polynucleotide of the present invention. In a preferred aspect, the dsRNA is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.

10 The dsRNA is preferably a small interfering RNA (siRNA) or a micro RNA (miRNA). In a preferred aspect, the dsRNA is small interfering RNA (siRNAs) for inhibiting transcription. In another preferred aspect, the dsRNA is micro RNA (miRNAs) for inhibiting translation.

15 The present invention also relates to such double-stranded RNA (dsRNA) molecules, comprising a portion of SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof for inhibiting expression of the polypeptide in a cell. While the present invention is not limited by any particular mechanism of action, the dsRNA can enter a cell and cause the degradation of a single-stranded RNA (ssRNA) of similar or identical sequences, including endogenous mRNAs. When a cell is exposed to dsRNA, mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi).

20 The dsRNAs of the present invention can be used in gene-silencing. In one aspect, the invention provides methods to selectively degrade RNA using a dsRNAi of the present invention. The process may be practiced *in vitro*, *ex vivo* or *in vivo*. In one aspect, the dsRNA molecules can be used to generate a loss-of-function mutation in a cell, an organ or an animal. Methods for making and using dsRNA molecules to selectively degrade RNA are well known in the art; see, for example, U.S. Patent Nos. 6,489,127; 6,506,559; 6,511,824; 25 and 6,515,109.

30 The present invention further relates to a mutant cell of a parent cell that comprises a disruption or deletion of a polynucleotide encoding the polypeptide or a control sequence thereof or a silenced gene encoding the polypeptide, which results in the mutant cell producing less of the polypeptide or no polypeptide compared to the parent cell.

35 The polypeptide-deficient mutant cells are particularly useful as host cells for the expression of native and heterologous polypeptides. Therefore, the present invention further relates to methods of producing a native or heterologous polypeptide, comprising: (a) cultivating the mutant cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. The term "heterologous polypeptides" means polypeptides that are not native to the host cell, e.g., a variant of a native protein. The host cell may

comprise more than one copy of a polynucleotide encoding the native or heterologous polypeptide.

The methods used for cultivation and purification of the product of interest may be performed by methods known in the art.

5

Methods of C4-Dicarboxylic Acid Production

The present invention is also directed to methods of using the polypeptides having C4-dicarboxylic acid transporter activity, or polynucleotides encoding the polypeptides having C4-dicarboxylic acid transporter activity. The C4-dicarboxylic acid transporters described herein or polynucleotides encoding the same may be used in a host cell (e.g., a filamentous fungal host cell) to aid in the production, such as increasing production, of a C4-dicarboxylic acid (e.g., malic acid). For these methods, any of the polynucleotides or polypeptides of the present invention described herein (e.g., SEQ ID NO: 1, 2, 3, 4, 5, and/or 6, or any described aspect thereof) may be used, as exemplified in the aspects set forth below.

15

In one aspect, the present invention relates to methods of producing a C4-dicarboxylic acid (e.g., malic acid), comprising: (1) cultivating a host cell (e.g., filamentous fungal host cell) comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (e.g., SEQ ID NO: 1, 3, 5, or any described aspect thereof), wherein the host cell secretes increased levels of the C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter; and (2) recovering the malic acid.

20

In another aspect, the present invention relates to methods for increasing C4-dicarboxylic acid production (e.g., malic acid production) relative to a parent host cell, comprising: (1) transforming into a host cell (e.g., a filamentous fungal host cell) a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter described herein (e.g., SEQ ID NO: 1, 3, 5, or any described aspect thereof), wherein the host cell secretes an increased level of C4-dicarboxylic acid compared to the filamentous fungal host cell without the heterologous polynucleotide; (2) cultivating the transformed organism in a medium; and (3) recovering the C4-dicarboxylic acid.

25

30

In some of these aspects of the methods, the C4-dicarboxylic acid is malic acid, succinic acid, oxaloacetic acid, malonic acid, or fumaric acid, or combinations thereof. In some aspects, the C4-dicarboxylic acid is malic acid, succinic acid, or fumaric acid, or combinations thereof. In some aspects, the C4-dicarboxylic acid is malic acid or fumaric acid, or a combination of malic acid and fumaric acid. In some aspects, the C4-dicarboxylic acid is malic acid.

35

As described *supra*, the C4-dicarboxylic acid transporter may be any C4-dicarboxylic acid transporters described herein, e.g., a C4-dicarboxylic acid transporter selected from: (a) a polypeptide having at least 60% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or a full-length complementary strand of the foregoing; (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof; (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, the mature polypeptide thereof; and (e) a fragment of the polypeptide of (a), (b), (c), or (d).

For example, in one aspect, the present invention relates to a method of producing a C4-dicarboxylic acid (e.g., malic acid), comprising:

(1) cultivating a host cell (e.g., filamentous fungal host cell) comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter, wherein the C4-dicarboxylic acid transporter is selected from: (a) a polypeptide having at least 60% sequence identity to SEQ ID NO: 2, or the mature polypeptide thereof; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 1, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 1, or the mature polypeptide coding sequence thereof; (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2 or the mature polypeptide sequence thereof; and (e) a fragment of the polypeptide of (a), (b), (c), or (d); wherein the host cell secretes increased levels of the C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter; and

(2) recovering the malic acid.

In another exemplary aspect, the present invention relates to a method of producing a C4-dicarboxylic acid (e.g., malic acid), comprising:

(1) cultivating a host cell (e.g., filamentous fungal host cell) comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter, wherein the C4-dicarboxylic acid transporter is selected from: (a) a polypeptide having at least 60% sequence identity to SEQ ID NO: 4 or the mature polypeptide thereof; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 3 the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 3 or the mature polypeptide coding sequence thereof; (d) a variant comprising a

substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 4 or the mature polypeptide sequence thereof; and (e) a fragment of the polypeptide of (a), (b), (c), or (d); wherein the host cell secretes increased levels of the C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter; and

(2) recovering the malic acid.

In another exemplary aspect, the present invention relates to a method of producing a C4-dicarboxylic acid (e.g., malic acid), comprising:

(1) cultivating a host cell (e.g., filamentous fungal host cell) comprising a heterologous polynucleotide encoding a C4-dicarboxylic acid transporter, wherein the C4-dicarboxylic acid transporter is selected from: (a) a polypeptide having at least 60% sequence identity to SEQ ID NO: 6 or the mature polypeptide thereof; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with SEQ ID NO: 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing; (c) a polypeptide encoded by a polynucleotide having at least 65% sequence identity to SEQ ID NO: 5 or the mature polypeptide coding sequence thereof; (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 6 or the mature polypeptide thereof; and (e) a fragment of the polypeptide of (a), (b), (c), or (d); wherein the host cell secretes increased levels of the C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide encoding a C4-dicarboxylic acid transporter; and

(2) recovering the malic acid.

Additional variations and embodiments of the polypeptides having C4-dicarboxylic acid transporter activity contemplated in the methods above are described throughout the present application.

In one aspect of the methods, the C4-dicarboxylic acid (e.g., malic acid) is produced or secreted at a titer greater than about 10 g/L, e.g., greater than about 25 g/L, 50 g/L, 75 g/L, 100 g/L, 125 g/L, 150 g/L, 160 g/L, 170 g/L, 180 g/L, 190 g/L, 200 g/L, 210 g/L, 225 g/L, 250 g/L, 275 g/L, 300 g/L, 325 g/L, 350 g/L, 400 g/L, or 500g/L; or between about 10 g/L and about 500 g/L, e.g., between about 50 g/L and about 350 g/L, about 100 g/L and about 300 g/L, about 150 g/L and about 250 g/L, about 175 g/L and about 225 g/L, or about 190 g/L and about 210 g/L.

In any of the aspects of the methods, the level of the produced or secreted C4-dicarboxylic acid (e.g., malic acid) in the host cell is increased by at least 25%, e.g., at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cell without the polynucleotide encoding the heterologous polynucleotide when cultivated under the same conditions.

In any of these aspects of the methods, the heterologous polynucleotide may be operably linked to a promoter foreign to the polynucleotide.

In any of these aspects of the methods, the host cell may further comprise a heterologous second polynucleotide encoding a malate dehydrogenase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 11, or any described aspect thereof) and/or a
5 heterologous third polynucleotide encoding a pyruvate carboxylase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 15, or any described aspect thereof), as described *supra*. In some aspects, the heterologous second and/or third polynucleotide is operably linked to a promoter foreign to the polynucleotide. Examples of malate
10 dehydrogenases and pyruvate carboxylases that may be used with these methods can be found, for example, in PCT Publication No. WO2011/028643, entitled "Methods for Improving Malic Acid Production in Filamentous Fungi" filed August 27, 2010, which describes the polynucleotides encoding malate dehydrogenase and pyruvate carboxylase polypeptides described therein.

15 In any of these aspects of the methods, the host cell may be any host cell described above, e.g., a filamentous fungal host cell, such as a host cell selected from the group consisting of an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*,
20 *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Rhizopus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolyptocladium*, *Trametes*, and *Trichoderma*. For example, the host cell may be an *Aspergillus* host cell, such as an *Aspergillus oryzae* host cell.

In the methods of the present invention, the recombinant host cell is cultivated in a nutrient medium suitable for production of the C4-dicarboxylic acid transporter, using
25 methods well known in the art, as described *supra*.

The recombinant C4-dicarboxylic acid can be optionally recovered from the fermentation medium using any procedure known in the art (see, for example, WO 1998/022611 and U.S. 7,601,865) including, but not limited to, chromatography (e.g., size
30 exclusion chromatography, adsorption chromatography, ion exchange chromatography), electrophoretic procedures, differential solubility, osmosis, distillation, extraction (e.g., liquid-liquid extraction), pervaporation, extractive filtration, membrane filtration, membrane separation, reverse, or ultrafiltration. In one example, the C4-dicarboxylic acid is recovered from other material in the fermentation medium by filtration.

In some aspects of the methods, the recombinant C4-dicarboxylic acid before and/or
35 after being optionally purified is substantially pure. With respect to the methods of producing

a C4-dicarboxylic acid (or a specific C4-dicarboxylic acid thereof, such as malic acid), "substantially pure" intends a recovered preparation of the C4-dicarboxylic acid that contains no more than 15% impurity, wherein impurity intends compounds other than C4-dicarboxylic acids. In one variation, a preparation of substantially pure C4-dicarboxylic acid is provided
5 wherein the preparation contains no more than 25% impurity, or no more than 20% impurity, or no more than 10% impurity, or no more than 5% impurity, or no more than 3% impurity, or no more than 1% impurity, or no more than 0.5% impurity.

Suitable assays to test for the production of C4-dicarboxylic acids for the methods of production and host cells described herein can be performed using methods known in the
10 art. For example, the final C4-dicarboxylic acid product (e.g., malic acid), and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of C4-dicarboxylic acid in the fermentation
15 broth can also be tested with the culture supernatant. Byproducts and residual sugar in the fermentation medium (e.g., glucose) can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., *Biotechnol. Bioeng.* 90:775 -779 (2005)), or using other suitable assay and detection methods well known in the art.

Signal Peptide

The present invention also relates to an isolated polynucleotide encoding a signal peptide. In one aspect, the signal peptide comprises or consists of amino acids 1 to 61 or 1 to 68 of SEQ ID NO: 2. In some aspects, the isolated polynucleotide encoding the signal
25 peptide is nucleotides 1 to 183 or 1 to 204 of SEQ ID NO: 1. In another aspect, the signal peptide comprises or consists of amino acids 1 to 17 of SEQ ID NO: 4. In some aspects, the isolated polynucleotide encoding the signal peptide is nucleotides 1 to 51 of SEQ ID NO: 3. In another aspect, the signal peptide comprises or consists of amino acids 1 to 68 of SEQ ID NO: 6. In some aspects, the isolated polynucleotide encoding the signal peptide is
30 nucleotides 1 to 204 of SEQ ID NO: 5.

The polynucleotides may further comprise a gene encoding a protein, which is operably linked to the signal peptide and/or propeptide. The protein is preferably foreign to the signal peptide and/or propeptide.

The present invention also relates to nucleic acid constructs, expression vectors and
35 recombinant host cells comprising such polynucleotides.

The present invention also relates to methods of producing a protein, comprising: (a) cultivating a recombinant host cell comprising such polynucleotide; and (b) recovering the protein.

5 The protein may be native or heterologous to a host cell. The term "protein" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "protein" also encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides and fused polypeptides.

10 Preferably, the protein is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. For example, the protein may be an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase such as an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, 15 invertase, laccase, another lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase or xylanase.

The gene may be obtained from any prokaryotic, eukaryotic, or other source.

20 The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

5

Fungal strains

Aspergillus aculeatus was used as source of the C4-dicarboxylic acid transport protein genes *c4t737*, *c4t521*, and *mat737*. *Aspergillus oryzae* NRRL 3488 (or ATCC 56747) was used as a source of a pyruvate carboxylase gene, a malate dehydrogenase gene, and for production of the C4-dicarboxylic acids.

10

Media

YEG medium was composed of 20 g glucose, 5 g yeast extract, and deionized water to 1 liter.

15

COVE plates were composed of 1 M sucrose, 2% COVE salt solution, 10 mM acetamide, 15 mM CsCl, and 25 g/l Agar Noble.

COVE salt solution was composed of 26 g KCl, 26 g MgSO₄·7H₂O, 76 g KH₂PO₄, 50 ml of COVE trace elements solution, and deionized water to 1 liter.

20

COVE trace elements solution was composed of 0.04 g Na₂B₄O₇·10H₂O, 0.04 g CuSO₄·5H₂O, 1.2 g FeSO₄·7H₂O, 0.7 g MnSO₄·H₂O, 0.8 g Na₂MoO₄·2H₂O, 10 g ZnSO₄·7H₂O and deionized water to 1 liter.

Seed medium was composed of 40 g glucose, 6 g Bacto-peptone, 750 mg KH₂PO₄, 750 mg K₂HPO₄, 100 mg MgSO₄·7H₂O, 100 mg CaCl₂·H₂O, 5 mg FeSO₄·7H₂O, 5 mg NaCl, and deionized water to 1 liter.

25

Seed medium B was composed of 30 g glucose, 3 g Bacto-peptone, 560 mg KH₂PO₄, 560 mg K₂HPO₄, 925 mg NaH₂PO₄·H₂O, 820 mg Na₂HPO₄, 75 mg MgSO₄·7H₂O, 75 mg CaCl₂·H₂O, 0.75 ml of 1000X Micronutrient Solution, and deionized water to 1 liter.

Acid production medium C was composed of 100 g glucose, 80 g CaCO₃, 6 g Bacto Peptone, 150 mg KH₂PO₄, 150 mg K₂HPO₄, 100 mg MgSO₄·7H₂O, 100 mg CaCl₂·H₂O, 1 ml 1000X Micronutrient Solution, and deionized water to 1 liter.

30

Fermentor batch medium was composed of 120 g glucose, 120 g CaCO₃, 9 g Bacto-peptone, 150 mg KH₂PO₄, 150 mg K₂HPO₄, 100 mg MgSO₄·7H₂O, 100 mg CaCl₂·2H₂O, 5 mg FeSO₄·7H₂O, 5 mg NaCl, 5 mL Pluronic L61, and deionized water to 1 liter.

1000X Micronutrient Solution was composed of 5 g NaCl, 5 g FeSO₄·7H₂O, 1 g citric acid, and deionized water to 1 liter.

35

PDA plates were composed of 39 g/l potato dextrose agar.

2XYT+amp plates were composed of 16 g tryptone, 10 g yeast extract, 5 g NaCl, 100 mg ampicillin, 15 g Bacto agar, and deionized water to 1 liter.

Example 1: Cloning of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene and construction of expression vector pSaMF35

Genomic DNA from *Aspergillus aculeatus* was isolated by inoculating 100 ml of YEG medium in a shake flask with 2×10^6 spores and incubating the flask at 34°C overnight with shaking at 160 rpm. The mycelia were harvested by filtration using a MIRACLOTH® (Calbiochem, San Diego, CA, USA) lined funnel and approximately 2 g of mycelia were recovered and frozen in liquid nitrogen. The frozen mycelia were disrupted by quickly smashing with a hammer while wrapped inside the MIRACLOTH®. The disrupted mycelia were then transferred to a 50 ml polypropylene conical centrifuge tube containing 10ml of 1X lysis buffer (100 mM EDTA, 10 mM Tris pH 8.0, 1% Triton® X-100, 0.5 M Guanidine-HCl, 200 mM NaCl) and 3 µl of RNase A (QIAGEN Inc., Valencia, CA, USA, 100 mg/ml). The tube was mixed by gentle vortexing, then incubated at room temperature for 5 minutes after which was added 150 µl Proteinase K (QIAGEN Inc., Valencia, CA, USA; 20 mg/ml). The tube was mixed by inversion and incubated at 50°C for 1 hour. The tube was then centrifuged at 7240 x g for 20 minutes. The supernatant was then added to a pre-equilibrated QIAGEN™-tip 100 (QIAGEN Inc., Valencia, CA, USA) and the remaining DNA extraction steps were performed according to the manufacturer's instructions. The DNA was resuspended in 100 µl TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0).

The 1194 bp C4-dicarboxylic acid transporter gene *c4t737* was amplified from *Aspergillus aculeatus* genomic DNA using primers 069698 and 069699 shown below.

Primer 069698:

5'-GTGATAGAACATCGTCCATAATGCTCGGGCAACACT-3' (SEQ ID NO: 7)

Primer 069699:

5'-GTGTCAGTCACCTCTAGTTATTACTCCGATACATCCTCGT-3' (SEQ ID NO: 8)

The PCR reaction was composed of 5 µl 10X reaction buffer (Stratagene, La Jolla, CA, USA), 1 µl *A. aculeatus* genomic DNA template (105 ng/µl), 1 µl primer 069698 (100 ng/µl), 1 µl primer 069699 (100 ng/µl), 1 µl dNTP mixture (10 mM), 40.5 µl deionized water, and 0.5 µl Herculase® HotStart DNA polymerase (Stratagene, La Jolla, CA, USA). The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® (Eppendorf Scientific Inc. Westbury, New York, USA) programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes; and 20 cycles each at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes plus 10 seconds per cycle. The PCR product was then purified using a MinElute® PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA).

Plasmid pShTh60 (Figure 1; see also PCT Publication No. WO2011/028643, filed August 27, 2010) was digested with *Sac* I and *Pac* I, separated by 0.8% agarose gel electrophoresis in TBE buffer (10.8 g/L Tris Base, 5.5 g/L Boric acid, 2 mM EDT A, pH 8.0) and purified using a QIAQUICK® Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA). The
5 purified PCR product above was then inserted into the digested pShTh60 fragment using an In-Fusion™ Advantage reaction kit (Clontech, Mountain View, CA, USA) composed of 2 µl 5X buffer (Clontech, Mountain View, CA, USA), 2.4 µl purified PCR product (33 ng/µl), 1.5 µl digested and gel-purified pShTh60 (132 ng/µl), 1 µl In-Fusion™ enzyme and 3.1 µl deionized water. The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, placed on ice
10 for 5 minutes and diluted with 40 µl TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0) resulting in pSaMF35 (Figure 2).

A 2.5 µl aliquot of the ligation reaction above containing pSaMF35 was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Transformants were plated onto 2XYT +amp
15 plates and incubated at 37°C overnight. The resulting transformants were picked and subjected to DNA sequencing to confirm that the *c4t737* gene was integrated into the vector.

The nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the *Aspergillus aculeatus* *c4t737* gene are shown in Figure 3. The coding sequence is 1194 bp including the stop codon. The encoded predicted protein is 397 amino acids, with a
20 predicted molecular mass of 44.3 kDa and an isoelectric pH of 6.93. The gene contains no introns. Using the SignalP program (Nielsen et al., 1997, *Protein Engineering* 10: 1-6), a signal peptide of 61 residues was predicted. Based on this program, the predicted mature protein contains 336 amino acids with a predicted molecular mass of 37.3 kDa and an isoelectric pH of 6.52. Using the InterProScan program (The European Bioinformatics
25 Institute), a signal peptide of 68 residues was predicted. Based on this program, the predicted mature protein contains 329 amino acids with a predicted molecular mass of 36.5 kDa and an isoelectric pH of 6.52.

Example 2: Transformation of an expression vector fragment of pSaMF35 into
30 ***Aspergillus oryzae* NRRL 3488 (SaMF35)**

Protoplast preparation and transformation of *Aspergillus oryzae* NRRL 3488 were performed by inoculating approximately 2×10^7 spores into 100 ml YEG medium and incubating the flask at 27°C for 16-18 hours at 140 rpm. Mycelia were collected by pouring the culture through a sterile funnel lined with MIRACLOTH® (Calbiochem, San Diego, CA, USA)
35 and rinsing with 50 ml of 0.7 M KCl. The washed mycelia were resuspended in a 125 ml flask containing 20 ml of protoplasting solution composed of 5 mg GLUCANEX™

(Novozymes A/S, Bagsværd, Denmark) and 0.5 mg chitinase (Sigma Chemical Co., St. Louis, MO, USA) per ml of 0.7 M KCl (filter sterilized) and incubated at 34°C for 30 minutes with mixing at 80 rpm. The protoplasting solution was poured through a sterile funnel lined with MIRACLOTH® and rinsed with 50 ml of STC buffer (1 M sorbitol-10 mM Tris-HCl pH 6.5-10 mM CaCl₂). The flow-through was collected in two 50 ml polypropylene tubes. The tubes were centrifuged at 1300 x g for 10 minutes at room temperature. The supernatant was discarded and the protoplast pellet was resuspended in 20 ml of STC buffer. The protoplasts were washed by two rounds of resuspending the pellet in 20 ml of STC buffer and centrifugation at 1300 x g for 10 minutes at room temperature. The final pellet was resuspended in 2 ml of STC buffer. The protoplasts were counted by removing a 10 µl sample and counting them in a hemacytometer (VWR, West Chester, PA, USA). The volume was adjusted with STC buffer to obtain a protoplast concentration of 2 x 10⁷ per ml.

Plasmid pSaMF35 was prepared for transformation by restriction digestion with *Pme* I. The 4977 bp expression cassette was separated from the digested vector by 0.8% agarose gel electrophoresis in TBE buffer and purified using a QIAQUICK® Gel Extraction Kit. Two transformation reactions were prepared. For each reaction, a 100 µl solution of protoplast preparation was transferred to a 12 ml polypropylene tube, to which was added 5 µg of linearized pSaMF35, 250 µl PEG solution (60% w/v polyethylene glycol (PEG), 10 mM Tris 6.5, 10 mM CaCl) followed by gentle mixing and incubation at 37°C for 30 minutes. Each transformation was diluted with 9 ml of STC buffer, followed by plating three separate 3 ml aliquots onto COVE plates. Each plate was then incubated at 34°C for 7-10 days. Twenty SaMF35 transformants were transferred to individual COVE plates and incubated at 34°C for 5 days. Spore stocks were prepared by collecting the spores in 0.1% TWEEN® 80. Cultures were stored by preparing a glycerol stock of each (800 µl spore stock, 200 µl 0.1% TWEEN® 80) and frozen at -80°C.

Example 3: Production of malic acid in shake flask cultures of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF35 (SaMF35)

Spores from each pSaMF35 transformant described in Example 2 and *Aspergillus oryzae* NRRL 3488 as a control were plated onto individual PDA plates and allowed to sporulate at 34°C for 5 to 7 days. Spores were collected in 0.1% TWEEN® 80 and counted using a hemacytometer. Seed cultures were prepared in 250 ml flasks containing 100 ml of seed medium B and inoculated with 300 µl of spore suspension. Seed cultures were grown for approximately 17 hours at 30°C with shaking at 200 rpm. Acid production cultures were prepared in 250 ml unbaffled flasks containing 50 ml of acid production medium C and 3 ml of the 17 hour seed cultures. Cultures were incubated at 30°C with shaking at 200 rpm for 2-10 days.

Quantitation of malic acid for the shake flask culture transformants was performed by Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) using an 1200 Series Binary LC System and 1200 Series Diode Array Detector (DAD) (Agilent Technologies, Santa Clara, CA USA). Reverse phase separation was performed using an Aqua 5 μ C18 125Å 205 x 4.6 mm ID column and AQ C18 4 x 3.0 mm Security Guard Cartridge (Phenomenex, Inc., Torrance, CA, USA). The mobile phase consisted of 10% methanol (HPLC grade) and 90% 145 mM phosphate pH 1.5 buffer.

Whole culture samples were removed and diluted 1:10 in HPLC Running Buffer composed of 850 ml of 64 mM phosphate buffer and 150 ml of methanol pH 1.65. The samples were then filtered through a 25 mm 0.45 micron polyethersulfone membrane (Whatman, Florham Park, NJ, USA) and 1.5 ml of the filtrates was placed into a HPLC vial for acid analysis. The remaining amount of the shake flask cultures were filtered through 3 layers of cheese cloth and rinsed three times with 10 volumes of double distilled sterile water to remove insoluble CaCO₃. Cell pellets were harvested from the cheese cloth, placed into a 15 ml culture tube and stored at -20°C.

RP-HPLC was performed using an injection volume of 10 μ l at a flow rate of 0.7 ml/minute (isocratic) with a column temperature of 25°C and run time of 11 minutes. Detection was set at 210 nm, 8 nm bandwidth, with the reference at 360 nm, 40 nm bandwidth. The void time was determined to be 3.8 minutes. The quantitative capabilities of the reverse phase method were determined for malic acid by performing replicate injections of serially diluted malic acid standards with concentrations ranging from 49.2-3.93 mM. The relative standard deviation for (RSD) for replicate injections was $\leq 5\%$. Malic acid shows $R^2 \geq 0.9999$.

Aspergillus oryzae transformant containing pSaMF35 showed an improvement in malic acid production of greater than 2-fold over the *Aspergillus oryzae* NRRL 3488 control strains after 3 days of shake flask growth.

Example 3B: Fermentation of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF35 (SaMF35)

Aspergillus oryzae pSaMF35 transformants described in Example 2 and control transformant *Aspergillus oryzae* ShTh1040 (see PCT Application No. PCT/US10/47002, filed August 27, 2010) were prepared and fermented as described in Example 7 below.

Quantitation of malic acid in the fermentations was performed as described above. The relative malic acid titer of *Aspergillus oryzae* pSaMF35 transformants were comparable to the *Aspergillus oryzae* ShTh1040 transformants, indicating that the *Aspergillus oryzae* pSaMF35 transformants outperform the *Aspergillus oryzae* NRRL 3488 control (which lack the overexpressed C4-dicarboxylic acid transporter gene) based on ShTh1040 and NRRL

3488 comparisons previously described.

Example 4: Cloning of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene and construction of expression vector pSaMF36

The 1257 bp C4-dicarboxylic acid transporter gene *c4t521* was amplified from isolated *Aspergillus aculeatus* genomic DNA (Example 1) using primers 069700 and 069701 shown below.

Primer 069700:

5'-TGTGATAGAACATCGTCCATAATGCACGACCACAGC-3' (SEQ ID NO: 9)

Primer 069701:

5'-GTGTCAGTCACCTCTAGTTATCATTCTGAACAACCTCGGACA-3' (SEQ ID NO: 10)

The PCR reaction was composed of 10 µl 5X reaction buffer, 1 µl *A. aculeatus* genomic DNA template (105 ng/µl), 1 µl primer 069700 (100 ng/µl), 1 µl primer 069701 (100 ng/µl), 1 µl dNTP mixture (10 mM), 35.5 µl deionized water, and 0.5 µl Phusion™ Hot Start High-Fidelity DNA polymerase (Finnzymes, Inc, Massachusetts, USA). The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 minute; and one cycle at 72°C for 10 minutes. The PCR product was digested with *Dpn* I for 1 hour to degrade any plasmid DNA template.

Plasmid pShTh60 (Figure 1) was digested with *Sex* AI and *Pac* I, separated by 0.8% agarose gel electrophoresis in TBE buffer, and purified using a QIAQUICK® Gel Extraction Kit. The purified PCR product above was then inserted into the digested pShTh60 fragment using an In-Fusion™ Advantage reaction kit composed of 2 µl 5X buffer, 3 µl purified PCR product (26 ng/µl), 1.5 µl gel-purified *Sex* AI and *Pac* I digested and gel-purified pShTh60 (132 ng/µl), 1 µl In-Fusion™ enzyme and 2.5 µl deionized water. The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes, placed on ice for 5 minutes and diluted with 40 µl TE buffer resulting in pSaMF36 (Figure 4).

A 2.5 µl aliquot of the ligation reaction above was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells according to the manufacturer's instructions. Transformants were plated onto 2XYT+amp plates and incubated at 37°C overnight. The resulting transformants were picked and subjected to DNA sequencing to confirm that the *mat521* gene was successfully integrated into the vector.

The nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of the *Aspergillus aculeatus c4t521* gene are shown in Figure 5. The coding sequence is 1257 bp including the stop codon. The encoded predicted protein is 418 amino acids, with a predicted molecular mass of 46.8 kDa and an isoelectric pH of 6.36. The gene contains no introns. Using the SignalP program (Nielsen et al., 1997, *Protein Engineering*

10: 1-6), a signal peptide of 17 residues was predicted. Based on this program, the predicted mature protein contains 401 amino acids with a predicted molecular mass of 44.9 kDa and an isoelectric pH of 6.89.

5 **Example 5: Transformation of an expression vector fragment of pSaMF36 into *Aspergillus oryzae* NRRL 3488 (SaMF36)**

Protoplast preparation and transformation of *Aspergillus oryzae* NRRL 3488 were performed as described in Example 2.

Plasmid pSaMF36 was prepared for transformation by restriction digestion with *Pme*
 10 I. The 5040 bp expression cassette was separated from the digested vector by 0.8% agarose gel electrophoresis in TBE buffer and purified using a QIAQUICK® Gel Extraction Kit. Two transformation reactions were prepared. For each transformation reaction, 100 µl of protoplast preparation was transferred to a 12 ml polypropylene tube, to which was added 5 µg of linearized pSaMF36 and 250 µl PEG solution (60% w/v polyethylene glycol (PEG),
 15 10 mM Tris 6.5, 10 mM CaCl) followed by gentle mixing and incubation at 37°C for 30 minutes. Each transformation was diluted with 9 ml of STC buffer, followed by plating three separate 3 ml aliquots onto COVE plates. Each plate was then incubated at 34°C for 7-10 days. Twenty SaMF36 transformants were transferred to individual COVE plates and incubated at 34°C for 5 days. Spore stocks were prepared by collecting the spores in 0.1%
 20 TWEEN® 80. Cultures were stored by preparing a glycerol stock of each (800 µl spore stock, 200 µl 0.1% TWEEN® 80) and frozen at -80°C.

Example 6: Production of malic acid in shake flask cultures of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF36 (SaMF36)

25 Spores from each pSaMF36 transformant described in Example 5 and *Aspergillus oryzae* NRRL 3488 as a control were prepared as described in Example 3. Quantitation of malic acid for the shake flask culture transformants was performed as described in Example 3.

Table 1 shows the relative increase in malic acid titer of transformants *Aspergillus oryzae* SaMF36-3 and *Aspergillus oryzae* SaMF36-4 compared to malic acid production of *Aspergillus oryzae* NRRL 3488 as a control after 3 days of shake flask growth. *Aspergillus oryzae* SaMF36-3 and *Aspergillus oryzae* SaMF36-4 produced an increase in malic acid titer of 2.1-fold and 2.3-fold, respectively, compared to *Aspergillus oryzae* NRRL 3488.

Table 1

Strain	Relative titer of malic acid	%CV
NRRL 3488	1	0.7%

SaMF36-3	2.1	4.8%
SaMF36-4	2.3	0.2%

Example 7: Fermentation of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF36 (SaMF36)

Aspergillus oryzae pSaMF36 transformants described in Example 5 and control transformant *Aspergillus oryzae* ShTh1040 (see PCT Application No. PCT/US10/47002, filed August 27, 2010) were grown for approximately 7 days at 34°C on PDA plates. A 5-6 ml volume of sterile 50 mM sodium phosphate buffer (pH 6.8) containing 0.2% TWEEN® 80 was added to each plate and spores were suspended by scraping with an inoculating loop. Each suspension was transferred by pipette to a 50 ml conical tube. For each tube, 25 ml of sterile sodium phosphate buffer (50 mM, pH 6.8) was added to a 500 ml unbaffled flask containing 75 ml of seed medium, which was then inoculated with 2 ml of spore suspension. The flasks were then incubated at 34°C and 180 rpm for about 24 hours. The seed flasks were combined to supply the 144 ml inoculum required per tank.

Three-liter fermentors containing 1.8 liters of fermentor batch medium were individually inoculated by introducing 144 ml (8%) of the seed culture broth from the combined seed flasks of either an *Aspergillus oryzae* pSaMF36 transformant or an *Aspergillus oryzae* ShTh1040 transformant. For this example only, the glucose concentration in the fermentor batch medium was reduced to 60 g/L and the feed start was delayed by one day. The fermentors were equilibrated at 34°C ± 0.1°C and stirred at 500 rpm. Inlet air flow was maintained at 1 v/v/m. A 20% glucose stream was administered at a rate of approximately 7.3 g/hr beginning at about 43 hours of fermentation. Sterile CaCO₃ (about 100 g) was added around day 5 or 6 to keep the fermentation pH in the range of 6 to 7. Samples were withdrawn daily and analyzed for malic acid production as described in Example 3. Fermentation was completed after 7 or 8 days.

Quantitation of malic acid in the fermentations was performed as described in Example 3. The relative malic acid titer of *Aspergillus oryzae* pSaMF36 transformants was comparable to *Aspergillus oryzae* ShTh1040, indicating that the *Aspergillus oryzae* pSaMF36 transformants outperform the *Aspergillus oryzae* NRRL 3488 (which lack the overexpressed C4-dicarboxylic acid transporter gene) in malic acid production based on ShTh1040 and NRRL 3488 comparisons previously described.

Example 8: Cloning of an *Aspergillus oryzae* malate dehydrogenase gene and construction of expression vector pSaMF21

Plasmid pSaMF21 was constructed to contain the NAD-dependent malate

dehydrogenase (*mdh3*) gene sequence (DOGAN: AO090701000013), a 1430 bp fragment from *Aspergillus oryzae* as described in PCT Publication No. WO2011/028643, filed August 27, 2010. The nucleotide sequence (SEQ ID NO: 11) and deduced amino acid sequence (SEQ ID NO: 12) of the *Aspergillus oryzae* NRRL 3488 malate dehydrogenase *mdh3* gene are shown in Figure 6. The genomic coding sequence of 1430 bp (including stop codon) encodes a polypeptide of 330 amino acids with a predicted mass of 35 kDa. The coding sequence is interrupted by 7 introns of 57 bp (14-70 bp), 70 bp (103-172 bp), 74 bp (284-357 bp), 68 bp (446-513 bp), 58 bp (892-949 bp), 48 bp (1035-1082 bp), and 62 bp (1228-1289 bp). The G +C content of the coding region of the *mdh3* gene is 50.3%.

Briefly, the plasmid was constructed by linearizing pShTh60 (Figure 1) by restriction digestion with *Sex* AI and *Pac* I. The digested vector was separated by 0.8% agarose gel electrophoresis in TBE buffer and purified using a QIAQUICK® Gel Extraction Kit. The *mdh3* gene was amplified from pShTh71 (PCT Application No. PCT/US10/47002, filed August 27, 2010) using primers 067522 and 067525.

Primer 067522:

5'-AGAACATCGTCCATAATGGTCAAAGCTGGTGAGTTA-3' (SEQ ID NO: 13)

Primer 067525:

5'-GTGTCAGTCACCTCTAGTTATTACTTTGGTGGTGGGTTCT-3' (SEQ ID NO: 14)

The PCR reaction was composed of 5 µl 10X reaction buffer, 1 µl pShTh71 template (87 ng/µl), 1 µl primer 067522 (100 ng/µl), 1 µl primer 067525 (100 ng/µl), 1 µl dNTP mixture (10 mM), 45.5 µl deionized water, and 0.5 µl Herculase® HotStart DNA polymerase (Stratagene, La Jolla, CA, USA). The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 1.5 minutes; 20 cycles each at 95°C for 10 seconds, 50°C for 30 seconds, and 72°C for 1.5 minutes plus 10 seconds per cycle. The PCR reaction was subjected to a restriction digest with *Dpn* I for 1 hour to degrade any plasmid DNA template. The PCR product was then purified using the MinElute® PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA). The purified PCR product was inserted into the vector using an In-Fusion™ Advantage reaction composed of 2 µl 5X buffer, 0.5 µl purified PCR product (110 ng/µl), 1.7 µl gel-purified *Sex* AI and *Pac* I restriction digested pShTh60 (Figure 1; 78 ng/µl), 1 µl In-Fusion™ enzyme and 4.8 µl deionized water. The reaction was incubated at 37°C for 15 minutes followed by 50°C for 15 minutes after which it was placed on ice for 5 minutes and diluted with 40 µl TE buffer resulting in pSaMF21 (Figure 7). A 2 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Transformants were plated onto 2XYT+amp plates and incubated at 37°C overnight. The resulting transformants were picked and

subjected to DNA sequencing to confirm that the *mdh3* gene was successfully integrated into the vector.

Example 9: Cloning of an *Aspergillus oryzae* pyruvate carboxylase gene and construction of expression vector pRyan1

Plasmid pRyan1 was constructed to contain the pyruvate carboxylase (*pyc*) gene sequence (DOGAN: AO090023000801), a 3646 bp fragment from *Aspergillus oryzae* (including two stop codons) as described in PCT Publication No. WO2011/028643, filed August 27, 2010. The nucleotide sequence (SEQ ID NO: 15) and deduced amino acid sequence (SEQ ID NO: 16) of the *Aspergillus oryzae* pyruvate carboxylase genes are shown in Figures 8A and 8B. Both the *Aspergillus oryzae* NRRL 3488 and ATCC 56747 pyruvate carboxylase genes have the same nucleotide sequence. The genomic coding sequence of 3643 bp (including one stop codon) encodes a polypeptide of 1193 amino acids with a predicted mass of 131 kDa. The coding sequence is interrupted by 1 intron of 61 bp (3475-3535 bp). The G+C content of the coding region of the gene is 57.1%.

Briefly, the plasmid was constructed by linearizing pShTh60 (Figure 1) by restriction digestion with *Sex* AI and *Pac* I. The digested vector was separated by 0.8% agarose gel electrophoresis in TBE buffer and purified using a QIAQUICK® Gel Extraction Kit. The *pyc* gene was amplified from *Aspergillus oryzae* NRRL 3488 genomic DNA using primers 066549 and 067388 shown below.

Primer 066549:

5'-TAGAACATCGTCCATAATGGCGGCTCCGTTTCGTCA-3' (SEQ ID NO: 17)

Primer 067388:

5'-GTGTCAGTCACCTCTAGTTATTATTACGCTTTGACGATCT-3' (SEQ ID NO: 18)

The PCR reaction was composed of 5 µl 10X reaction buffer, 1 µl *Aspergillus oryzae* NRRL3488 genomic DNA (110 ng/µl), 1 µl primer 066549 (100 ng/µl), 1 µl primer 067388 (100 ng/µl), 1 µl dNTP mixture (10 mM), 45.5 µl deionized water, and 0.5 µl Herculase® HotStart DNA polymerase. The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 3.5 minutes; 20 cycles each at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 3.5 minutes plus 10 seconds per cycle. The PCR product was then purified using a MinElute® PCR Purification Kit.

The purified PCR product was inserted into the vector using an In-Fusion™ Advantage reaction composed of 2 µl 5X buffer, 1 µl purified PCR product (144 ng/µl), 2 µl gel purified *Sex* AI and *Pac* I restriction digested pShTh60 (Figure 1; 78 ng/µl), 1 µl In-Fusion™ enzyme and 4 µl deionized water. The reaction was incubated at 37°C for 15 minutes followed by 50°C for 15 minutes after which it was placed on ice for 5 minutes and

diluted with 40 µl TE buffer resulting in pRYAN1 (Figure 9). A 2 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells according to the manufacturer's instructions. Transformants were plated onto 2XYT+amp plates and incubated at 37°C overnight. The resulting transformants were picked and subjected to DNA sequencing to confirm that the *pyc* gene was successfully integrated into the vector. Nucleotide 1308 was changed from C to T, but did not affect the protein sequence.

Example 10: Transformation of expression vector fragments of pSaMF36, pSaMF21 and pRyan1 into *Aspergillus oryzae* NRRL 3488 (SaMF3603)

The vectors pSaMF36 (Example 4), pSaMF21 (Example 8) and pRyan1 (Example 9) were prepared for transformation by digestion with *Pme* I for 4 hours at 37°C. The digested vectors were separated on a 0.8% agarose TBE gel, a 5040 bp band was cut out for pSaMF36, a 5213 bp band was cut out for pSaMF21 and a 7429 bp band was cut out for pRyan1. The bands containing the expression cassettes were each purified using the Macherey-Nagel Nucleospin® Extract II Kit (Düren, Germany) according to manufacturer's instructions.

Three transformation reactions were prepared. For each transformation reaction, 100 µl of protoplast preparation (Example 2) were transferred to a 12 ml polypropylene tube. To this was added a total of five micrograms of amp marker free, linearized pShTh104, pSaMF21 and pRyan1 in equimolar quantities and 250 µl of polyethylene glycol (PEG) solution (60% w/v polyethylene glycol (PEG), 10 mM Tris 6.5, 10 mM CaCl) followed by gentle mixing and incubation at 37°C for 30 minutes. Each transformation was diluted with 9 ml of STC buffer, followed by plating three separate 3ml aliquots onto COVE plates. Each plate was then incubated at 34°C for 7-10 days. The resulting transformants were transferred to individual COVE plates and incubated at 34°C for 5 days. Spore stocks were prepared by collecting the spores in 0.1% TWEEN® 80. Cultures were stored by preparing a glycerol stock of each (800 µl spore stock, 200 µl 0.1% TWEEN® 80) and frozen at -80°C.

Example 11: Production of malic acid in shake flask cultures of *Aspergillus oryzae* transformants containing expression vector fragments of pSaMF36, pSaMF21 and pRyan1 (SaMF3603)

Spores from each *Aspergillus oryzae* triple transformant SaMF3603 described in Example 10 and *Aspergillus oryzae* NRRL 3488 as a control were prepared as described in Example 3. Quantitation of malic acid for the shake flask culture transformants was performed as described in Example 3.

Aspergillus oryzae transformants SaMF3603 containing pSaMF36, pSaMF21 and pRyan1 showed an improvement in malic acid production of greater than 2.55-fold over the *Aspergillus oryzae* NRRL 3488 control strains.

5 **Example 12: Fermentation of *Aspergillus oryzae* transformants containing expression vector fragments of pSaMF36, pSaMF21 and pRyan1 (SaMF3603)**

Aspergillus oryzae triple transformant SaMF3603 described in Example 10 and control transformant *Aspergillus oryzae* SaMF2103 (see PCT Application No. PCT/US10/47002, filed August 27, 2010) were prepared and fermented as described in
10 Example 7.

Quantitation of malic acid in the fermentations was performed as described in Example 3. The relative malic acid titer of *Aspergillus oryzae* triple transformants SaMF3603 were comparable to the *Aspergillus oryzae* transformants SaMF2103, indicating that the *Aspergillus oryzae* triple transformants SaMF3603 outperform both the *Aspergillus oryzae*
15 pSaMF36 single transformants and the *Aspergillus oryzae* NRRL 3488 control based on comparisons previously described.

Example 13: Cloning of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene and construction of expression vector pSaMF38

20 The 1194 bp C4-dicarboxylic acid transporter gene *mat737* was synthetically constructed into pAaMAT737 (Figure 10; DNA2.0, Menlo Park, CA, USA). The *mat737* gene was amplified from pAaMAT737 using primers 069698 and 069699 (Example 1).

The PCR reaction was composed of 10 µl 5X reaction buffer, 1 µl pAaMAT737 template (20 ng/µl), 1 µl primer 069698 (100 ng/µl), 1 µl primer 069699 (100 ng/µl), 1 µl
25 dNTP mixture (10 mM), 35.5 µl deionized water, and 0.5 µl Phusion® Hot Start High-Fidelity DNA polymerase. The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 98°C for 30 seconds; 30 cycles each at 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 1 minute; 1 cycle at 72°C for 10 minutes. The PCR reaction was digested with *Dpn* I for 1 hour to degrade any plasmid DNA template
30 and the PCR product was purified using the MinElute® PCR Purification Kit.

Plasmid pShTh60 (Figure 1) was digested with *Sex* AI and *Pac* I, separated by 0.8% agarose gel electrophoresis in TBE buffer (10.8 g/L Tris Base, 5.5 g/L Boric acid, 2 mM EDTA, pH 8.0) and purified using a QIAQUICK® Gel Extraction Kit. The purified PCR product above was then inserted into the digested pShTh60 fragment using an In-Fusion™
35 Advantage reaction kit composed of 2 µl 5X buffer, 0.5 µl purified PCR product (187 ng/µl), 1.5 µl digested and gel-purified pShTh60 (132 ng/µl), 1 µl In-Fusion™ enzyme and 5 µl deionized water. The reaction was incubated at 37°C for 15 minutes, 50°C for 15 minutes,

placed on ice for 5 minutes and diluted with 40 µl TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0) resulting in pSaMF38 (Figure 11).

A 2.5 µl aliquot of the ligation reaction above containing pSaMF38 was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells according to the manufacturer's instructions. Transformants were plated onto 2XYT+amp plates and incubated at 37°C overnight. The resulting transformants were picked and subjected to DNA sequencing to confirm that the *mat737* gene was integrated into the vector.

The nucleotide sequence (SEQ ID NO: 5) and deduced amino acid sequence (SEQ ID NO: 6) of the *mat737* gene are shown in Figure 12. The coding sequence is 1194 bp including the stop codon. The encoded predicted protein is 397 amino acids, with a predicted molecular mass of 44.3 kDa and an isoelectric pH of 7.32. The gene contains no introns. Using the InterProScan program (The European Bioinformatics Institute), a signal peptide of 68 residues was predicted. Based on this program, the predicted mature protein contains 329 amino acids with a predicted molecular mass of 36.6 kDa and an isoelectric pH of 6.52.

Example 14: Transformation of an expression vector fragment pSaMF38 into *Aspergillus oryzae* NRRL 3488 (SaMF38)

Protoplast preparation and transformation of *Aspergillus oryzae* NRRL 3488 were performed as described in Example 2.

Plasmid pSaMF38 was prepared for transformation by restriction digestion with *Pme* I. The 4977 bp expression cassette was separated from the digested vector by 0.8% agarose gel electrophoresis in TBE buffer and purified using a Macherey-Nagel Nucleospin Extract II Kit. Two transformation reactions were prepared. For each transformation reaction, 100 µl of protoplast preparation was transferred to a 12 ml polypropylene tube, to which was added 5 µg of linearized pSaMF38 and 250 µl PEG solution (60% w/v polyethylene glycol (PEG), 10 mM Tris 6.5, 10 mM CaCl) followed by gentle mixing and incubation at 37°C for 30 minutes. Each transformation was diluted with 9 ml of STC buffer, followed by plating three separate 3 ml aliquots onto COVE plates. Each plate was then incubated at 34°C for 7-10 days. Twenty SaMF38 transformants were transferred to individual COVE plates and incubated at 34°C for 5 days. Spore stocks were prepared by collecting the spores in 0.1% TWEEN® 80. Cultures were stored by preparing a glycerol stock of each (800 µl spore stock, 200 µl 0.1% TWEEN® 80) and frozen at -80°C.

Example 15: Production of malic acid in shake flask cultures of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF38 (SaMF38)

Spores from *Aspergillus oryzae* pSaMF38 transformants described in Example 14

and *Aspergillus oryzae* NRRL 3488 as a control were prepared as described in Example 3. Quantitation of malic acid for the shake flask culture transformants was performed as described in Example 3.

5 *Aspergillus oryzae* pSaMF38 transformant showed an improvement in malic acid production of greater than 1.8-fold over the *Aspergillus oryzae* NRRL 3488 control strains.

Example 16: Cloning of an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene and construction of expression vector pSaMF41 under control of an alternative promoter

10 The following examples demonstrate that an *Aspergillus aculeatus* C4-dicarboxylic acid transporter gene can be driven by using an alternative *gpd* promoter.

The 1257 bp C4-dicarboxylic acid transporter gene *c4t521* was amplified from pSaMF36 (*supra*) using primers 0611384 and 069701 shown below.

Primer 0611384:

15 5'- CCAACAGACACATCTAAACAATGCACGACCACAGCA-3' (SEQ ID NO: 21)

Primer 069701:

5'- GTGTCAGTCACCTCTAGTTATCATTCGAACAACTCGGACA-3' (SEQ ID NO: 22)

20 The PCR reaction was composed of 5 µl 10X reaction buffer, 1 µl pSaMF36 template (50 ng/µl), 1 µl primer 0611384 (100 ng/µl), 1 µl primer 069701 (100 ng/µl), 1 µl dNTP mixture (10 mM), 40.5 µl deionized water, and 0.5 µl Herculase® HotStart DNA polymerase (Stratagene, La Jolla, CA, USA). The amplification reaction was incubated in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 95°C for 2 minutes; 10 cycles each at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes; 20 cycles each at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes plus 10
25 seconds per cycle. The PCR reaction was subjected to restriction digestion with *DpnI* for 1 hour to degrade any plasmid DNA template. The PCR product was then purified using the MinElute® PCR Purification Kit (QIAGEN Inc.).

30 The purified PCR product was then inserted into a vector containing the *gpd* promoter (pShTh108) using an In-Fusion™ Advantage reaction composed of 2 µl 5X buffer, 0.6 µl purified PCR product (127 ng/µl), 1.75 µl gel-purified *Hind* III and *Pac* I restriction digested pShTh108 (114 ng/µl), 1 µl In-Fusion™ enzyme and 4.65 µl deionized water. The reaction was incubated at 37°C for 15 minutes then 50°C for 15 minutes, and then placed on ice for 5 minutes and diluted with 40 µl TE buffer resulting in pSaMF41.

35 A 2.5 µl aliquot of the ligation reaction was transformed into ONE SHOT® TOP10 chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated onto 2XYT+amp plates and incubated at 37°C overnight. The

resulting transformants were picked and subjected to DNA sequencing to confirm that the *c4t521* gene was successfully integrated into the vector.

The nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of the *Aspergillus aculeatus c4t521* gene are shown in Figure 5. The coding sequence is 1257 bp including the stop codon and is driven by the *gpd* promoter compared to the *pgk* promoter of Example 4.

Example 17: Transformation of an expression vector fragment pSaMF41 into *Aspergillus oryzae* NRRL 3488 (SaMF41)

Protoplast preparation and transformation of *Aspergillus oryzae* 3488 were performed as described in Example 2.

Plasmid pSaMF41 was prepared for transformation by linearizing by restriction digestion with *Pme* I. The 5025 bp expression cassette was separated from the digested vector by 0.8% agarose gel electrophoresis in TBE buffer and purified using a Macherey-Nagel Nucleospin® Extract II Kit for gel isolation according to manufacturer's instructions. Two transformation reactions were prepared. For each transformation reaction, 100 µl of protoplast preparation was transferred to a 12 ml polypropylene tube. To this was added 5 µg of linearized pSaMF41 and 250 µl PEG solution (60% w/v polyethylene glycol (PEG), 10 mM Tris 6.5, 10 mM CaCl) followed by gentle mixing and incubation at 37°C for 30 minutes. Each transformation was diluted with 9 ml of STC buffer, followed by plating three separate 3 ml aliquots onto COVE plates. Each plate was then incubated at 34°C for 7-10 days. SaMF41 transformants were transferred to individual COVE plates and incubated at 34°C for 5 days. Spore stocks were prepared by collecting the spores in 0.1% TWEEN® 80. Cultures were stored by preparing a glycerol stock of each (800 µl spore stock, 200 µl 0.1% TWEEN® 80) and frozen at -80°C.

Example 18: Production of malic acid in shake flask cultures of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF41 (SaMF41)

Spores from each pSaMF41 transformant (SaMF41) described in Example 17, pSaMF36 transformant (SaMF36) described in Example 5, and *Aspergillus oryzae* NRRL 3488 as a control were prepared as described in Example 3. Quantitation of malic acid for the shake flask culture transformants was performed as described in Example 3.

Aspergillus oryzae SaMF41 transformants containing *Aspergillus aculeatus c4t521* gene (SEQ ID NO: 3) driven by the *gpd* promoter showed comparable malic acid production to *Aspergillus oryzae* SaMF36 transformants containing *Aspergillus aculeatus c4t521* gene (SEQ ID NO: 3) driven by the *pgk* promoter, and an increase in malic acid titer of about 2-fold compared to *Aspergillus oryzae* NRRL 3488 control lacking the *c4t521* gene.

Example 19: Fermentation of *Aspergillus oryzae* transformants containing an expression vector fragment of pSaMF41 (SaMF41)

Aspergillus oryzae pSaMF41 transformants described in Example 18 and control transformant *Aspergillus oryzae* ShTh1040 (see PCT Application No. PCT/US10/47002, filed August 27, 2010) were prepared and fermented as described in Example 7.

Quantitation of malic acid in the fermentations was performed as described above. The relative malic acid titer of *Aspergillus oryzae* pSaMF41 transformants were comparable to the *Aspergillus oryzae* ShTh1040 transformants, indicating that the *Aspergillus oryzae* pSaMF41 transformants outperform the *Aspergillus oryzae* NRRL 3488 control (which lack the overexpressed C4-dicarboxylic acid transporter gene) based on ShTh1040 and NRRL 3488 comparisons previously described.

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, IL, USA, and given the following accession number:

Deposit	Accession Number	Date of Deposit
<i>Escherichia coli</i> pAaC4T737	NRRL B-50400	June 17, 2010
<i>Escherichia coli</i> pAaC4T521	NRRL B-50388	June 4, 2010
<i>Escherichia coli</i> pAaMAT737	NRRL B-50401	June 17, 2010

The strain has been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by foreign patent laws to be entitled thereto. The deposit represents a substantially pure culture of the deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The present invention may be further described by the following numbered paragraphs:

[1] An isolated polypeptide having C4-dicarboxylic acid transporter activity, selected from:

(a) a polypeptide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof;

5 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

10 (c) a polypeptide encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof;

15 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

20

[2] An isolated polypeptide having C4-dicarboxylic acid transporter activity, selected from:

(a) a polypeptide having at least 75%, e.g., at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or the mature polypeptide sequence thereof;

25 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

30 (c) a polypeptide encoded by a polynucleotide having at least 75%, e.g., at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or the mature polypeptide coding sequence thereof;

35

(d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2 or the mature polypeptide sequence thereof; and

5 (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

[3] An isolated polypeptide having C4-dicarboxylic acid transporter activity, selected from:

10 (a) a polypeptide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof;

15 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 3, the mature polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

20 (c) a polypeptide encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or the mature polypeptide coding sequence thereof;

25 (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 4 or the mature polypeptide sequence thereof; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

30 [4] An isolated polypeptide having C4-dicarboxylic acid transporter activity, selected from:

(a) a polypeptide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 6 or the mature polypeptide sequence thereof;

35 (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 5, the mature

polypeptide coding sequence thereof, or the full-length complementary strand of the foregoing;

(c) a polypeptide encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5 or the mature polypeptide coding sequence thereof;

(d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 6 or the mature polypeptide sequence thereof; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has C4-dicarboxylic acid transporter activity.

[5] The polypeptide of any one of paragraphs [1] - [4], having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof.

[6] The polypeptide of any one of paragraphs [1] - [5], which is encoded by a polynucleotide that hybridizes under low stringency conditions, low-medium stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1, 3, or 5, the mature polypeptide coding sequence thereof, or the full-length complementary strand thereof.

[7] The polypeptide of any one of paragraphs [1] - [6], which is encoded by a polynucleotide having at least 65%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 1, 3, or 5, or the mature polypeptide coding sequence thereof.

[8] The polypeptide of any one of paragraphs [1] - [7], comprising or consisting of SEQ ID NO: 2, 4, or 6.

[9] The polypeptide of any one of paragraphs [1] - [7], comprising or consisting of the mature polypeptide of SEQ ID NO: 2, 4, or 6.

[10] The polypeptide of paragraph [9], wherein the mature polypeptide of SEQ ID NO: 2 is amino acids 62 to 397 or 69 to 397 of SEQ ID NO: 2.

5 [11] The polypeptide of paragraph [9], wherein the mature polypeptide of SEQ ID NO: 4 is amino acids 18 to 418 of SEQ ID NO: 4.

[12] The polypeptide of paragraph [9], wherein the mature polypeptide of SEQ ID NO: 6 is amino acids 69 to 397 of SEQ ID NO: 6.

10

[13] The polypeptide of any one of paragraphs [1] - [7], which is a fragment of SEQ ID NO: 2, 4, or 6, wherein the fragment has C4-dicarboxylic acid transporter activity.

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[14] The polypeptide of any one of paragraphs [1] - [7], which is a variant comprising a substitution, deletion, and/or insertion of one or more (e.g., two, several) amino acids of SEQ ID NO: 2, 4, or 6, or the mature polypeptide sequence thereof.

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[15] The polypeptide of any one of paragraphs [1] - [13], which is encoded by the polynucleotide contained in plasmid pAaC4T737 which is contained in *E. coli* NRRL B-50400.

[16] The polypeptide of any one of paragraphs [1] - [13] which is encoded by the polynucleotide contained in plasmid pAaC4t521 which is contained in *E. coli* NRRL B-50388.

25

[17] The polypeptide of any one of paragraphs [1] - [13], which is encoded by the polynucleotide contained in plasmid pAaMAT737 which is contained in *E. coli* NRRL B-50401.

30

[18] A composition comprising the polypeptide of any one of paragraphs [1] - [17].

[19] An isolated polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17].

35

[20] A nucleic acid construct or expression vector comprising the polynucleotide of paragraph [19] operably linked to one or more (e.g., two, several) control sequences that direct the production of the polypeptide in an expression host.

[21] A recombinant host cell comprising the polynucleotide of paragraph [19] operably linked to one or more control sequences that direct the production of the polypeptide.

5 [22] A method of producing the polypeptide of any one of paragraphs [1] - [17], comprising:

- (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

10 [23] A method of producing the polypeptide of any one of paragraphs [1] - [17], comprising:

- (a) cultivating the recombinant host cell of paragraph [21] under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

15

[24] A transgenic plant, plant part or plant cell transformed with a polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17].

20 [25] A method of producing the polypeptide of any one of paragraphs [1] - [17], comprising:

- (a) cultivating the transgenic plant or the plant cell of paragraph [24] under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

25 [26] A method of producing a mutant of a parent cell, comprising inactivating a polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17], which results in the mutant producing less of the polypeptide than the parent cell.

30 [27] A mutant cell produced by the method of paragraph [26].

[28] The mutant cell of paragraph [27], further comprising a gene encoding a native or heterologous protein.

[29] A method of producing a protein, comprising:

- 35
- (a) cultivating the mutant cell of paragraph [27] or [28] under conditions conducive for production of the protein; and
 - (b) recovering the protein.

[30] A double-stranded inhibitory RNA (dsRNA) molecule comprising a subsequence of the polynucleotide of paragraph [19], wherein the dsRNA is optionally an siRNA or an miRNA molecule.

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[31] The double-stranded inhibitory RNA (dsRNA) molecule of paragraph [30], which is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.

10 [32] A method of inhibiting the expression of a polypeptide having C4-dicarboxylic acid transporter activity in a cell, comprising administering to the cell or expressing in the cell the double-stranded RNA (dsRNA) molecule of paragraph [30] or [31].

[33] A cell produced by the method of paragraph [31] or [32].

15 [34] The cell of paragraph [33], further comprising a gene encoding a native or heterologous protein.

[35] A method of producing a protein, comprising:

- 20 (a) cultivating the cell of paragraph [33] or [34] under conditions conducive for production of the protein; and
(b) recovering the protein.

[36] An isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 61 or 1 to 68 of SEQ ID NO: 2.

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[37] An isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 17 of SEQ ID NO: 4.

30 [38] An isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids 1 to 68 of SEQ ID NO: 6.

[39] A nucleic acid construct or expression vector comprising a gene encoding a protein operably linked to the polynucleotide of any of paragraphs [36] - [38], wherein the gene is foreign to the polynucleotide encoding the signal peptide.

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[40] A recombinant host cell comprising the nucleic acid construct or expression vector of paragraph [39].

[41] A method of producing a protein, comprising:

(a) cultivating a recombinant host cell of paragraph [40] under conditions conducive for production of the protein; and

5 (b) recovering the protein.

[42] A method of producing a C4-dicarboxylic acid, comprising:

(a) cultivating a host cell comprising a heterologous polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17] in a medium; and

10 (b) recovering the C4-dicarboxylic acid.

[43] A method for increasing C4-dicarboxylic acid production, comprising:

(a) transforming into a host cell a heterologous polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17];

15 (b) cultivating the transformed organism in a medium; and

(c) recovering the C4-dicarboxylic acid.

[44] The method of paragraph [42] or [43], wherein the heterologous polynucleotide is operably linked to a promoter foreign to the polynucleotide.

20

[45] The method of any one of paragraphs [42] - [44], wherein the host cell further comprises a heterologous second polynucleotide encoding a malate dehydrogenase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 11, or any described aspect thereof).

25

[46] The method of paragraph [45], wherein the heterologous second polynucleotide is operably linked to a promoter foreign to the polynucleotide.

[47] The method of any one of paragraphs [42] - [46], wherein the host cell further comprises a heterologous third polynucleotide encoding a pyruvate carboxylase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 15, or any described aspect thereof).

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[48] The method of paragraph [47], wherein the heterologous third polynucleotide is operably linked to a promoter foreign to the polynucleotide.

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[49] The method of any one of paragraphs [42] - [48], wherein the host cell is a filamentous fungal host cell.

[50] The method of paragraph [49], wherein the filamentous fungal host cell is selected from an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*,
5 *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Rhizopus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, and *Trichoderma*.

[51] The method of paragraph [50], wherein the host cell is an *Aspergillus* host cell.

[52] The method of paragraph [50], wherein the host cell is an *Aspergillus oryzae* host cell.

[53] The method of any one of paragraphs [42] - [52], wherein the medium is a fermentable medium.

[54] The method of any one of paragraphs [42] - [53], wherein the C4-dicarboxylic acid is at a titer greater than about 10 g/L, e.g., greater than about 25 g/L, 50 g/L, 75 g/L, 100 g/L, 125 g/L, 150 g/L, 160 g/L, 170 g/L, 180 g/L, 190 g/L, 200 g/L, 210 g/L, 225 g/L, 250 g/L, 275 g/L, 300 g/L, 325 g/L, 350 g/L, 400 g/L, or 500g/L; or between about 10 g/L and about 500 g/L, e.g., between about 50 g/L and about 350 g/L, about 100 g/L and about 300 g/L, about 150 g/L and about 250 g/L, about 175 g/L and about 225 g/L, or about 190 g/L and about 210 g/L.

[55] The method of any one of paragraphs [42] - [54], wherein the level of the C4-dicarboxylic acid is increased by at least 25%, e.g., at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cell without the polynucleotide encoding the C4-dicarboxylic acid transporter when cultivated under the same conditions.

[56] The method of any one of paragraphs [42] - [55], wherein the C4-dicarboxylic acid is selected from malic acid, succinic acid, oxaloacetic acid, malonic acid, and fumaric acid.

[57] The method of paragraph [56], wherein the C4-dicarboxylic acid is malic acid.

[58] A host cell comprising a heterologous polynucleotide encoding the polypeptide of any one of paragraphs [1] - [17]; wherein the host cell is capable of secreting an increased level

of C4-dicarboxylic acid compared to the host cell without the heterologous polynucleotide when cultivated under the same conditions.

5 [59] The host cell of paragraph [58], wherein the heterologous polynucleotide is operably linked to a promoter foreign to the polynucleotide.

10 [60] The host cell of paragraph [57] or [58], further comprising a heterologous second polynucleotide encoding a malate dehydrogenase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 11, or any described aspect thereof).

[61] The host cell of paragraph [60], wherein the heterologous second polynucleotide is operably linked to a promoter foreign to the polynucleotide.

15 [62] The host cell of any one of paragraphs [58] - [61], further comprising a heterologous third polynucleotide encoding a pyruvate carboxylase (e.g., the mature polypeptide coding sequence of SEQ ID NO: 15, or any described aspect thereof).

20 [62] The host cell of paragraph [62], wherein the heterologous third polynucleotide is operably linked to a promoter foreign to the polynucleotide.

[63] The host cell of any one of paragraphs [58] - [62], wherein the host cell is a filamentous fungal host cell.

25 [64] The filamentous fungal host cell of paragraph [63], wherein the host cell is selected from an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Rhizopus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, and *Trichoderma*.

30 [65] The filamentous fungal host cell of paragraph [64], wherein the host cell is an *Aspergillus* host cell.

35 [66] The filamentous fungal host cell of paragraph [64], wherein the host cell is an *Aspergillus oryzae* host cell.

[67] The host cell of any one of paragraphs [58] - [66], wherein the cell is capable of C4-dicarboxylic acid volumetric productivity greater than about 0.1 g/L per hour, e.g., greater than about 0.2 g/L per hour, 0.5 g/L per hour, 0.6 g/L per hour, 0.7 g/L per hour, 0.8 g/L per hour, 0.9 g/L per hour, 1.0 g/L per hour, 1.1 g/L per hour, 1.2 g/L per hour, 1.3 g/L per hour, 1.5 g/L per hour, 1.75 g/L per hour, 2.0 g/L per hour, 2.25 g/L per hour, 2.5 g/L per hour, or 3.0 g/L per hour; or between about 0.1 g/L per hour and about 2.0 g/L per hour, e.g., between about 0.3 g/L per hour and about 1.7 g/L per hour, about 0.5 g/L per hour and about 1.5 g/L per hour, about 0.7 g/L per hour and about 1.3 g/L per hour, about 0.8 g/L per hour and about 1.2 g/L per hour, or about 0.9 g/L per hour and about 1.1 g/L per hour.

[68] The host cell of any one of paragraphs [58] - [67], wherein the host cell is capable of secreting an increased level of the C4-dicarboxylic acid of at least 25%, e.g., at least 50%, at least 100%, at least 150%, at least 200%, at least 300%, or at 500% compared to the host cell without the polynucleotide encoding the heterologous first polynucleotide when cultivated under the same conditions.

[69] The host cell of any one of paragraphs [58] - [68], wherein the C4-dicarboxylic acid is selected from malic acid, succinic acid, oxaloacetic acid, malonic acid, and fumaric acid.

[70] The host cell of paragraph [69], wherein the C4-dicarboxylic acid is malic acid.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Claims

What is claimed is:

1. An isolated polypeptide having C4-dicarboxylic acid transporter activity, selected from:
 - (a) a polypeptide having at least 80% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof; or
 - (b) a polypeptide encoded by a polynucleotide having at least 80% sequence identity to SEQ ID NO: 3 or the mature polypeptide coding sequence thereof;wherein the mature polypeptide sequence is amino acids 18 to 418 of SEQ ID NO: 4 and wherein the mature polypeptide coding sequence is nucleotides 52 to 1257 of SEQ ID NO: 3.
2. The polypeptide of claim 1, having at least 85% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
3. The polypeptide of claim 1, having at least 90% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
4. The polypeptide of claim 1, having at least 91% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
5. The polypeptide of claim 1, having at least 92% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
6. The polypeptide of claim 1, having at least 93% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
7. The polypeptide of claim 1, having at least 94% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
8. The polypeptide of claim 1, having at least 95% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
9. The polypeptide of claim 1, having at least 96% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

10. The polypeptide of claim 1, having at least 97% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
11. The polypeptide of claim 1, having at least 98% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
12. The polypeptide of claim 1, having at least 99% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
13. The polypeptide of claim 1, having 100% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
14. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 85% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
15. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 90% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
16. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 91% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
17. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 92% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
18. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 93% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
19. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 94% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
20. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 95% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
21. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 96% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.

22. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 97% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
23. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 98% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
24. The polypeptide of claim 1, which is encoded by a polynucleotide having at least 99% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
25. The polypeptide of claim 1, which is encoded by a polynucleotide having 100% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
26. The polypeptide of any one of claims 1-25, comprising or consisting of SEQ ID NO: 4.
27. The polypeptide of any one of claims 1-25, comprising or consisting of the mature polypeptide of SEQ ID NO:4.
28. A composition comprising the polypeptide of any one of claims 1-27 and a medium.
29. An isolated polynucleotide encoding the polypeptide of any one of claims 1-27.
30. A nucleic acid construct or expression vector comprising the polynucleotide of claim 29 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
31. A recombinant host cell comprising the polynucleotide of claim 29 operably linked to one or more control sequences that direct the production of the polypeptide.
32. A host cell comprising a heterologous polynucleotide that encodes a C4-dicarboxylic acid transporter, wherein the heterologous polynucleotide:
- (a) encodes a C4-dicarboxylic acid transporter having at least 80% sequence identity to SEQ ID NO:4 or the mature polypeptide sequence thereof; or
 - (b) has at least 80% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof;
- wherein the host cell produces a greater amount of C4 dicarboxylic acid compared to the host cell without the heterologous polynucleotide when cultivated under the same conditions; and

wherein the mature polypeptide sequence is amino acids 18 to 418 of SEQ ID NO: 4 and wherein the mature polypeptide coding sequence is nucleotides 52 to 1257 of SEQ ID NO: 3.

33. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 85% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

34. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 90% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

35. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 91% sequence identity to SEQ ID NO: 4, or the mature polypeptide sequence thereof.

36. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 92% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

37. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 93% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

38. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 94% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

39. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 95% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

40. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 96% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.

41. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 97% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
42. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 98% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
43. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having at least 99% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
44. The host cell of claim 32, wherein the heterologous polynucleotide encodes a C4-dicarboxylic acid transporter having 100% sequence identity to SEQ ID NO: 4 or the mature polypeptide sequence thereof.
45. The host cell of claim 32, wherein the heterologous polynucleotide has at least 85% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
46. The host cell of claim 32, wherein the heterologous polynucleotide has at least 90% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
47. The host cell of claim 32, wherein the heterologous polynucleotide has at least 91% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
48. The host cell of claim 32, wherein the heterologous polynucleotide has at least 92% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
49. The host cell of claim 32, wherein the heterologous polynucleotide has at least 93% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
50. The host cell of claim 32, wherein the heterologous polynucleotide has at least 94% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
51. The host cell of claim 32, wherein the heterologous polynucleotide has at least 95% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.

52. The host cell of claim 32, wherein the heterologous polynucleotide has at least 96% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
53. The host cell of claim 32, wherein the heterologous polynucleotide has at least 97% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
54. The host cell of claim 32, wherein the heterologous polynucleotide has at least 98% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
55. The host cell of claim 32, wherein the heterologous polynucleotide has at least 99% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
56. The host cell of claim 32, wherein the heterologous polynucleotide has 100% sequence identity to SEQ ID NO:3 or the mature polypeptide coding sequence thereof.
57. The host cell of any one of claims 32-56, wherein the heterologous polynucleotide is operably linked to a promoter foreign to the polynucleotide.
58. The host cell of any one of claims 32-57, further comprising a heterologous second polynucleotide encoding a malate dehydrogenase.
59. The host cell of claim 58, further comprising a heterologous third polynucleotide encoding a pyruvate carboxylase.
60. The host cell of any one of claims 32-59, wherein the host cell is a filamentous fungal host cell.
61. The filamentous fungal host cell of claim 60, wherein the host cell is an *Aspergillus* host cell.
62. The filamentous fungal host cell of claim 60, wherein the host cell is an *Aspergillus oryzae* host cell.
63. The host cell of any one of claims 32-62, wherein the C4-dicarboxylic acid is malic acid.

64. A method of producing a C4-dicarboxylic acid, comprising:
- (a) cultivating the host cell of any one of claims 32-63 in a medium under suitable conditions to produce a C4-dicarboxylic acid; and
 - (b) recovering the C4-dicarboxylic acid.
65. A method for increasing C4-dicarboxylic acid production in a host cell, comprising:
- (a) transforming into the host cell a heterologous polynucleotide encoding the polypeptide of any one of claims 1-27;
 - (b) cultivating the transformed host cell in a medium under suitable conditions to produce a C4-dicarboxylic acid; and
 - (c) recovering the C4-dicarboxylic acid.

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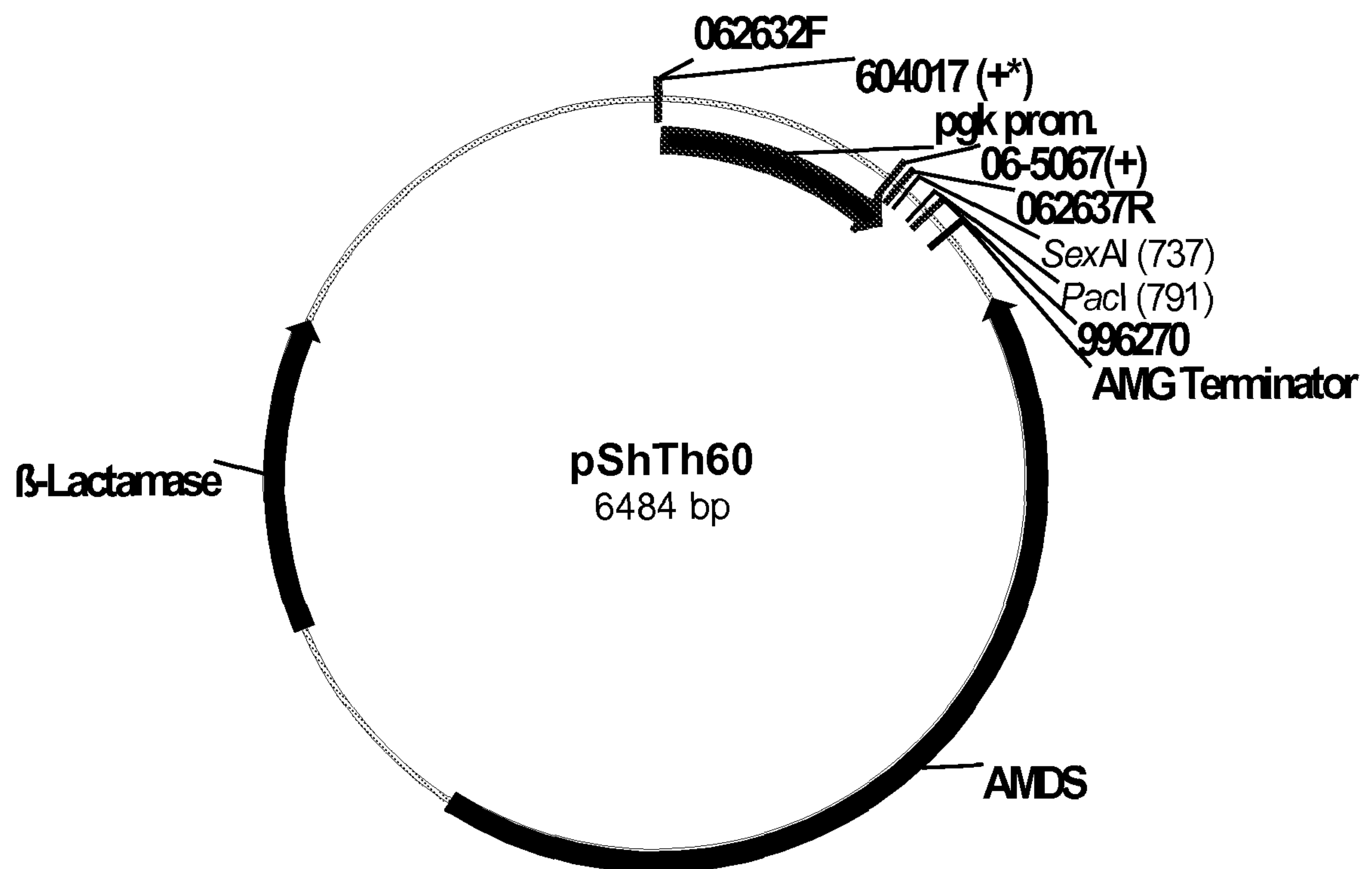


Fig. 1

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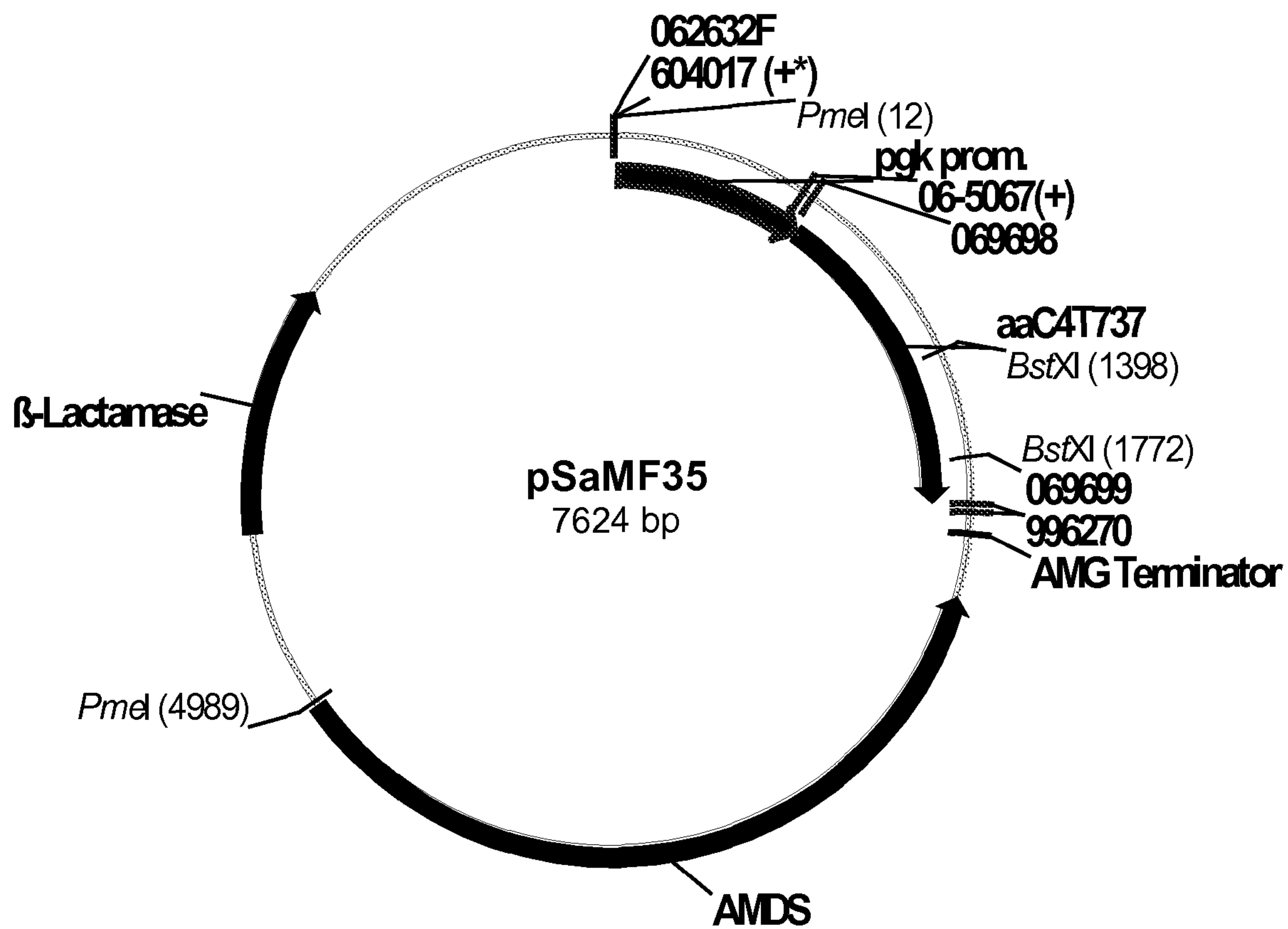


Fig. 2

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M L G Q H P P P D T S C S D L T T Y Q H
1 ATGCTCGGGC AACATCCGCC TCCCGACACC TCCTGCTCGG ACCTTACAAC ATACCAGCAT
E L K A S K Y S S S T N V S L R D R L R
61 GAGCTCAAAG CCTCCAAATA CTCTAGTTCC ACCAATGTGT CTCTACGGGA CCGTCTGCGT
H F T W A W Y T L T M S T G G L A L L L
121 CATTTTACCT GGGCGTGGTA TACTCTGACT ATGAGCACCG GCGGTCTAGC CCTCCTGCTG
A S Q P Y S F S G L Q Q I G L A V Y I I
181 GCCAGCCAGC CCTACTCCTT CTCCGGACTG CAACAGATCG GGCTTGCAGT CTACATCATC
N L A F F A L L C S L M A A R F I L H G
241 AACCTGGCCT TCTTTGCGTT GCTGTGTAGC CTCATGGCCG CACGCTTCAT TCTCCACGGC
N F L D S L R H D R E G L F F P T F W L
301 AACTTCCTCG ACTCCCTCCG ACACGACCGC GAGGGTCTTT TCTTTCCTAC TTTCTGGCTT
S I A T I I T G L Y R Y F G D T T Q P A
361 TCTATTGCAA CTATCATCAC CGGCCTGTAC CGCTACTTCG GCGACACCAC ACAGCCTGCA
F I Y A L E V L F W L Y C A F T L M T A
421 TTCATTTACG CTCTTGAGGT GCTCTTCTGG CTCTACTGTG CCTTCACTCT GATGACCGCT
I I Q Y S F V F T A H H Y P L Q T M M P
481 ATTATCCAAT ACTCCTTTGT CTTTACCGCC CACCACTACC CTCTACAAAC GATGATGCCC
S W I L P A F P I M L S G T I A S V I A
541 TCATGGATCC TCCCCGCATT CCCTATCATG CTCAGCGGCA CGATCGCCTC CGTCATTGCC
E Q Q P A R S A I P M I V A G T T F Q G
601 GAACAGCAGC CCGCGCGCTC TGCTATTCCC ATGATCGTCG CCGGCACCAC CTTCCAAGGC
L G F S I S F L M Y A H Y I G R L M E T
661 CTTGGCTTCT CCATCAGTTT CCTCATGTAC GCGCACTATA TCGGGCGGCT CATGGAGACG
G L P S R E H R P G M F I C V G P P A F
721 GGCCTTCCGT CCCGGGAACA CCGACCCGGG ATGTTTCATCT GCGTTGGCCC CCCGGCTTTC
T A L A L I G M T N G L P E D F Q V L Q
781 ACGGCCCTTG CCCTAATCGG CATGACCAAC GGCCTTCCTG AGGATTTTCA AGTCCTTCAA
D P H P F Q D A H I L R L L A I A T G A
841 GACCCGCACC CCTTTCAAGA CGCGCACATC CTCCGACTCC TTGCCATCGC CACGGGCGCC
F L W A L S L W F F S I A I I A T I R L
901 TTCCTCTGGG CCCTCAGTCT CTGGTTCTTT AGCATTGCCA TCATCGCCAC CATCCGCCTC
P P T A F H L N W W A M V F P N T G F T
961 CCACCTACAG CCTTCCACCT CAACTGGTGG GCCATGGTTT TTCCAAACAC GGGTTTTACT
L A T I T L G K A F D S P G V K G V G S
1021 CTCGCGACCA TCACGCTGGG CAAAGCCTTC GATAGCCCTG GAGTCAAGGG CGTCGGATCT
A M S I C I V G M W L F V F A S N I R A
1081 GCCATGTCCA TTTGCATCGT GGGGATGTGG CTGTTCGTGT TTGCGAGCAA TATCCGTGCC
V V K R D I V F P G K D E D V S E *
1141 GTTGTCAAAC GGGATATTGT TTTCCCTGGG AAGGACGAGG ATGTATCGGA GTAA

Fig. 3

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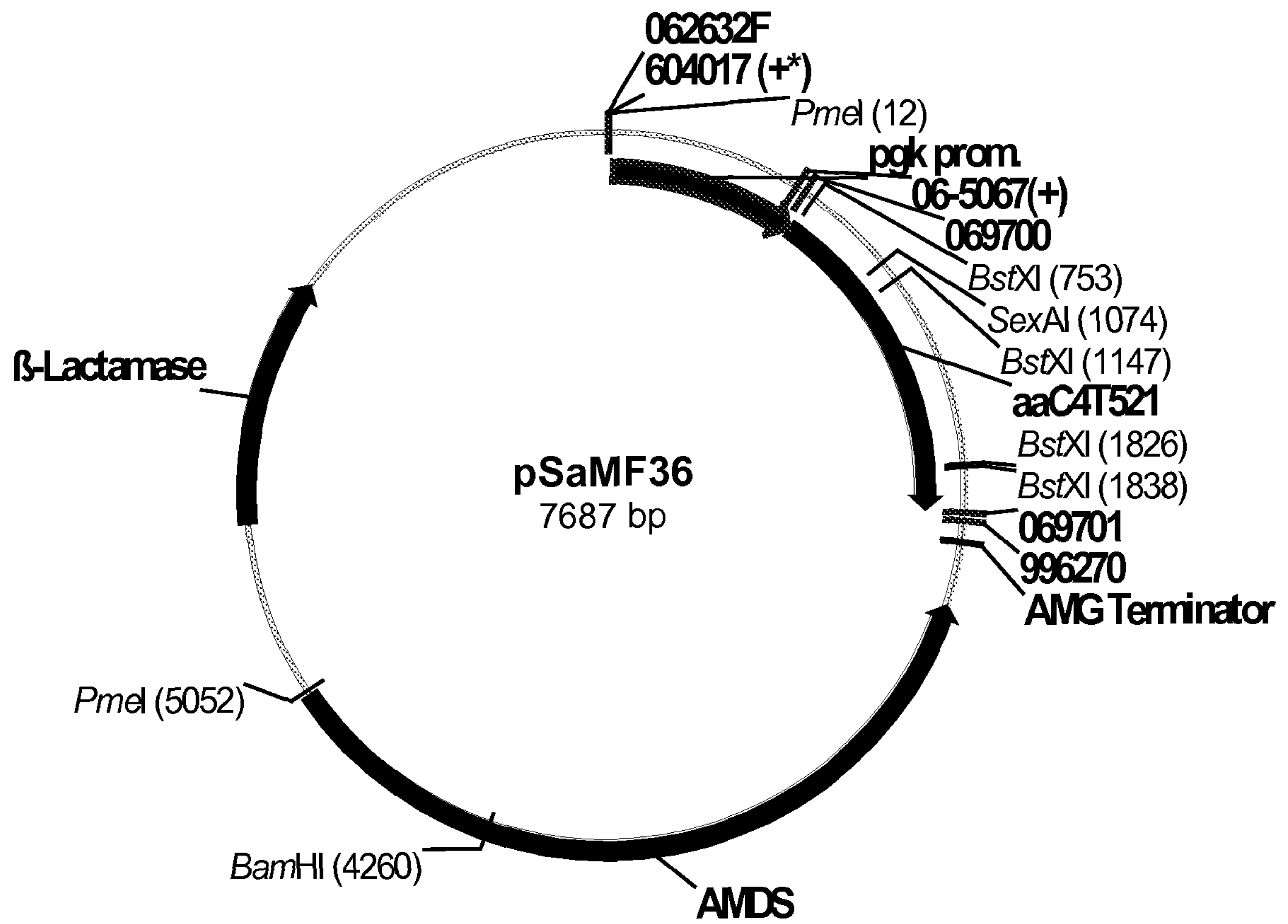


Fig. 4

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      M H D H S T G S S P Y I S D V E T L N H
1  ATGCACGACC ACAGCACTGG ATCTAGTCCA TACATCTCGG ACGTGGAAC CTTGAACCAC
      A C E K S V N P E T K V S Q P Q E S P I
61 GCCTGCGAGA AGTCCGTCAA CCCCAGAGACC AAAGTCTCCC AGCCTCAGGA ATCTCCCATT
      I S N N E H Q E F V K L G I R Q R L R H
121 ATCAGCAATA ATGAACATCA GGAGTTTGTT AAGCTGGGCA TCCGCCAACG GCTGCGTCAT
      F T W A W Y T L T M S A G G L A L L L R
181 TTCACCTGGG CCTGGTATAC CCTAACCATG AGCGCAGGTG GACTGGCCCT TCTTCTCCGC
      N Q P Y Q F K G L K E I G L V V Y I A N
241 AACCAGCCGT ATCAATTCAA GGGGTTGAAG GAGATAGGCC TGGTGGTATA CATAGCCAAT
      L V F F T I I G S L M I T R F V L Y N N
301 CTCGTCTTCT TTACTATCAT CGGCTCTCTT ATGATCACCA GGTTTGTTCT TTACAACAAC
      L M D S L R H D R E G F F F P T F W L S
361 CTGATGGACT CTCTCCGCCA CGACCGAGAA GGTTCCTTCT TTCCAACCTT CTGGCTCTCC
      I A T M I S G L S A Y F S T E D T H R L
421 ATCGCCACCA TGATTAGTGG TCTATCTGCC TACTTCTCTA CTGAAGACAC GCACCGCCTC
      N Y A L E G L F W A Y C I F T F A S A V
481 AATTATGCTC TCGAGGGTCT CTTCTGGGCG TACTGTATCT TCACGTTTGC CTCAGCAGTG
      I Q Y S F V F S Y H T F P L Q T M M P S
541 ATCCAGTACT CCTTTGTCTT CTCCTATCAC ACGTTCCTC TGCAAACCTAT GATGCCATCA
      W I L P A F P I M L S G T I A S A A S S
601 TGGATCTTAC CGGCATTCCC TATCATGCTG AGCGGAACCA TTGCCTCTGC CGCTTCCAGC
      Y Q P A V S A T P M I V A G I T F Q G L
661 TACCAGCCTG CGGTGTCTGC CACGCCTATG ATTGTTGCCG GCATCACGTT CCAGGGACTC
      G F C I S F M M Y A H Y I G R L M E T G
721 GGATTCTGCA TCAGCTTCAT GATGTACGCC CACTACATCG GGCGTCTGAT GGAGACGGGC
      I P S S E H R P G M F I C V G P P A F T
781 ATCCCTTCGA GCGAGCACCG TCCTGGTATG TTCATCTGTG TCGGCCCCC TGCCTTCACG
      L L A I I G M A N G L P E G F S I L G D
841 CTGCTGGCTA TCATCGGCAT GGCCAACGGC CTTCCCGAGG GCTTCAGTAT CCTGGGCGAT
      G G M D D R H I M R V L A V C A G M F L
901 GGTGGCATGG ACGACCGTCA CATCATGCGA GTACTGGCCG TCTGCGCGGG CATGTTCTCTC
      W A L S I W F F C V A L G S V V R A P P
961 TGGGCTCTGA GCATTTGGTT CTTCTGTGTC GCTCTGGGCT CAGTTGTGCG GGCGCCTCCC
      H D F H L N W W A M V F P N T G L T L A
1021 CATGATTTCC ACCTCAACTG GTGGGCTATG GTCTTCCCTA ACACCGGACT CACTCTCGCC
      T I T L A K S L D S A A L K W V G V G M
1081 ACCATCACCC TGGCCAAGTC ACTGGACAGT GCCGCGTTGA AATGGGTGGG CGTGGGCATG
      S L C V I C M F I F V F V S T I R A V L
1141 TCCCTCTGCG TGATCTGCAT GTTCATCTTC GTCTTCGTGA GCACCATTAG GGCTGTTCTC
      L K R I M W P G R D E D V S E L F E *
1201 TTGAAGAGGA TCATGTGGCC AGGTCGGGAT GAGGATGTGT CCGAGTTGTT CGAATGA

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Fig. 5

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      M V K A
1  ATGGTCAAAG CTGGTGAGTT AGCAATCCTT AACAGATGAC ACTCTCATAG GTACTAACTC
      A V L G A S G G I G Q
61  GAAACGTTAG CGGTACTTGG AGCTTCTGGT GGCATTGGCC AGGTATGGAT ATCCCCACGC
      P L S .
121 CTTACAACCC TGGTCACAAT ATGACCTTGT TCGATACTGA CTATCTCCCA AGCCACTGTC
      . L L L K T C P L V E E L A L Y D V V N T .
181 TCTCCTGTTG AAGACCTGTC CCTTAGTTGA AGAGCTTGCT CTCTACGATG TTGTGAACAC
      . P G V A A D L S H I S S I A
241 CCCTGGTGTG GCTGCTGATC TATCCACAT CTCGTCTATC GCTGTACGTT ACTGCCACAA
      K
301 TGCGAATTGC CCGATGGAAG AGGCGAAAAA TGGTATCTTG CTTACCTGGG CGATTAGAAA
      I S G F L P K D D G L K Q A L T G A N I
361 ATCTCTGGTT TTCTGCCCAA AGATGATGGG CTGAAGCAGG CCCTTACTGG TGCTAATATT
      V V I P A G I P
421 GTTGTCAATC CGGCTGGTAT TCCCCGTAAG TCCCTACCCT TTCGCATTGC TCCTCGTATG
      R K P G M T R D D L .
481 TTCGCTGGTG GCCAGTTTTT TGATAGTTGA TAGGCAAGCC TGGTATGACC CGTGACGACC
      . F K I N A G I V R D L V K G I A E F C P .
541 TCTTCAAGAT CAACGCCGGC ATAGTGCGAG ACTTGGTCAA GGGTATCGCC GAGTTCTGCC
      . K A F V L V I S N P V N S T V P I A A E .
601 CCAAGGCCTT TGTTCTGGTT ATCTCAAACC CCGTTAATTC TACTGTTTCT ATTGCTGCAG
      . V L K A A G V F D P K R L F G V T T L D .
661 AGGTGCTCAA AGCCGCTGGC GTCTTTGACC CGAAGCGCCT CTTTGGTGTC ACCACACTGG
      . V V R A E T F T Q E F S G Q K D P S A V .
721 ACGTCGTTTC TGCAGAGACT TTCACCCAAG AGTTCTCGGG CCAGAAGGAT CCTTCTGCTG
      . Q I P V V G G H S G E T I V P L F S K T .
781 TTCAAATCCC AGTTGTTGGT GGCCACTCTG GAGAGACCAT TGTCCCCCTC TTCAGCAAGA
      . T P A I Q I P E E K Y D A L I H
841 CTACCCCCGC AATTCAGATA CCCGAGGAGA AGTATGACGC ACTGATCCAC CGTAGGTTGT
      R V Q F
901 CCCAAAGAAT CTCATGAATA TCTTGCTGTA AGCACTAACT ATGCTTCAGG CGTCCAATTT
      G G D E V V Q A K D G A G S A T L S M A
961 GGTGGAGATG AGGTGGTCCA AGCTAAGGAC GGTGCTGGTT CCGCCACCTT GTCTATGGCC
      Y A G Y
1021 TATGCCGGTT ACAGGTAGGG ATGCTGCGTA CCGTGAGAGC ACTCGCGGCT AACATGCCAT
      R F A E S V I K A S K G Q T G I V E P T
1081 AGGTTGCTG AGAGTGTAAT CAAAGCTTCA AAGGGTCAAA CGGGTATTGT CGAGCCTACC
      F V Y L P G I P G G D E I V K A T G V E
1141 TTCGTCTACC TGCCTGGAAT TCCCGGCGGT GATGAGATCG TTAAGGCAAC TGGCGTGGAA
      F F S T L V T L G
1201 TTCTTCTCTA CTCTTGTAAC CTTAGGAGTA AGATTCATCT CCTCACAGAA TCTTCGTTCA
      T N G A E K A S N V L .
1261 TATCACGCCA GGCTAACGCT ATTAACAGA CTAATGGCGC AGAGAAGGCT AGCAACGTTT
      . E G V T E K E K K L L E A C T K G L K G .
1321 TTGAGGGCGT GACCGAGAAG GAAAAGAAGC TTCTCGAGGC TTGCACGAAA GGCCTTAAGG
      . N I E K G I D F V K N P P P K *
1381 GTAATATCGA GAAAGGCATC GACTTCGTTA AGAACCCACC ACCAAAGTAA

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Fig. 6

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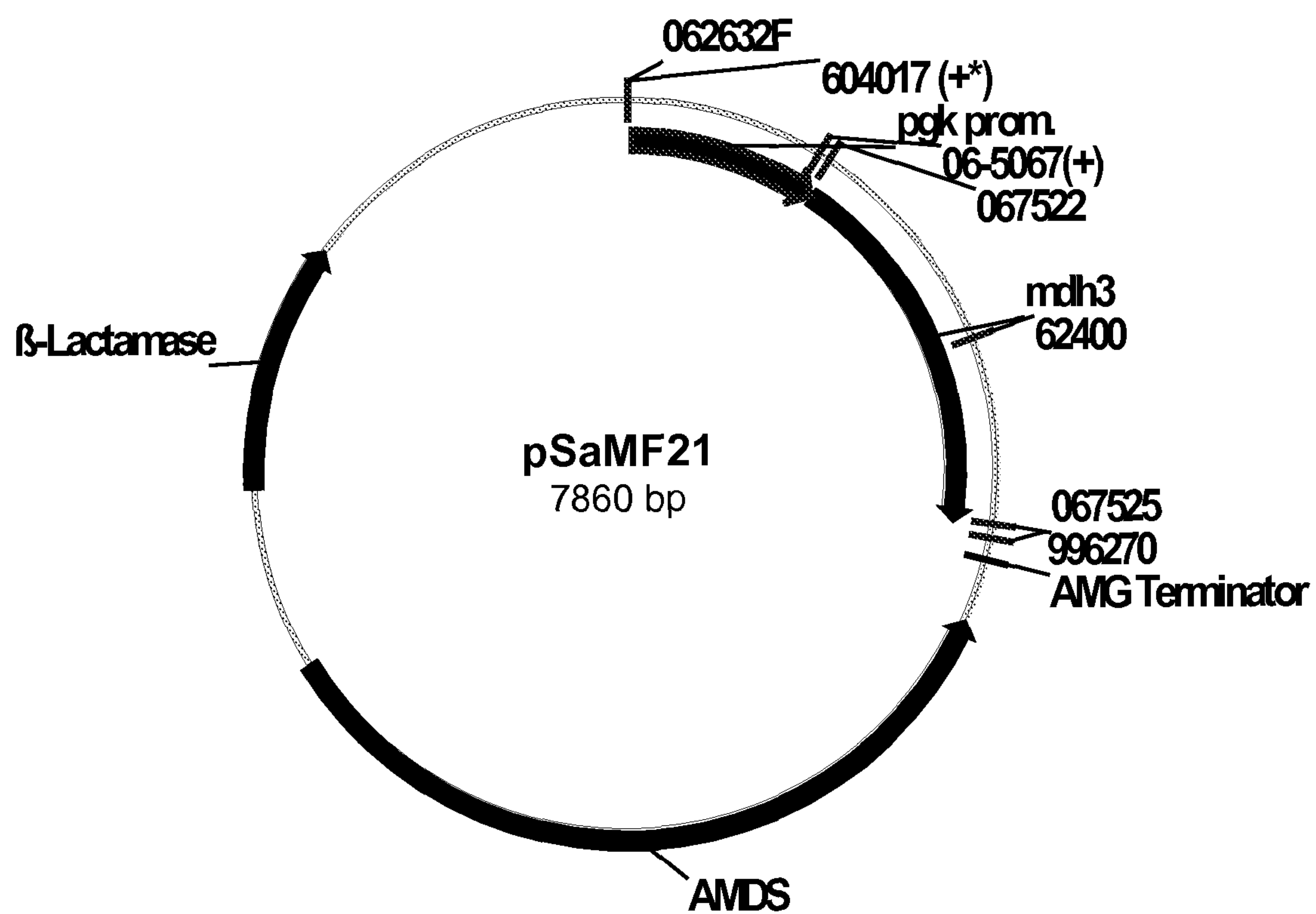


Fig. 7

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M A A P F R Q P E E A V D D T E F I D D
1 ATGGCGGCTC CGTTTCGTCA GCCTGAGGAG GCGGTTCGATG ACACCGAGTT CATCGATGAC
H H E H L R D T V H H R L R A N S S I M
61 CACCATGAAC ACCTCCGTGA TACCGTGCAC CATCGGTTGC GCGCCAATTC CTCCATTATG
H F Q K I L V A N R G E I P I R I F R T
121 CACTTCCAGA AGATCCTCGT CGCCAACCGT GGTGAGATCC CCATTTCGTAT CTTCAGAACG
A H E L S L Q T V A I Y S H E D R L S M
181 GCCCACGAGC TGTCCTTGCA GACGGTTGCT ATCTACTCTC ATGAGGATCG ACTGTCAATG
H R Q K A D E A Y M I G H R G Q Y T P V
241 CACCGTCAAA AGGCCGATGA GGCCTACATG ATTGGCCACC GCGGTCAGTA CACCCCTGTC
G A Y L A G D E I I K I A L E H G V Q L
301 GGTGCGTACC TGGCGGGCGA TGAGATCATC AAGATCGCCC TGGAGCACGG TGTCCAGCTG
I H P G Y G F L S E N A D F A R K V E N
361 ATCCACCCGG GCTACGGTTT CTTGTCCGAG AACGCCGACT TCGCCCGCAA GGTTGAGAAC
A G I V F V G P T P D T I D S L G D K V
421 GCCGGCATTG TCTTTGTGGG ACCCACTCCC GATACCATTG ACAGCTTGGG TGACAAGGTG
S A R R L A I K C E V P V V P G T E G P
481 TCGGCCCCGTC GGCTGGCCAT TAAGTGCAG GTCCTGTCTG TTCCGGGTAC GGAGGGCCCC
V E R Y E E V K A F T D T Y G F P I I I
541 GTCGAGCGCT ATGAGGAGGT CAAGGCGTTC ACAGACACCT ATGGCTTCCC CATCATCATC
K A A F G G G G R G M R V V R D Q A E L
601 AAGGCTGCCT TTGGCGGTGG TGGCCGTGGT ATGCGTGTGG TCCGTGACCA GGCCGAGCTG
R D S F E R A T S E A R S A F G N G T V
661 CGTGA CTCTG TCGAGCGAGC CACCTCTGAG GCCCGCTCCG CCTTCGGCAA TGGTACCGTC
F V E R F L D K P K H I E V Q L L G D S
721 TTCGTGAGC GCTTCCTCGA CAAACCCAAG CACATTGAAG TCCAGCTTCT GGGTGACAGC
H G N V V H L F E R D C S V Q R R H Q K
781 CACGGCAACG TTGTCCATCT GTTTGAGCGT GACTGCTCCG TGCAGCGTCG TCACCAGAAG
V V E V A P A K D L P A D V R D R I L A
841 GTCGTTGAGG TTGCTCCGGC TAAGGACCTG CCAGCCGATG TCCGGGACCG CATCCTGGCC
D A V K L A K S V N Y R N A G T A E F L
901 GATGCTGTGA AGCTGGCCAA GTCCGTCAAC TACCGTAACG CCGGTACAGC TGAGTTCCTG
V D Q Q N R H Y F I E I N P R I Q V E H
961 GTGGACCAGC AGAACCGCCA CTACTTCATT GAAATCAATC CTCGTATCCA AGTCGAGCAC
T I T E E I T G I D I V A A Q I Q I A A
1021 ACCATCACCG AAGAGATTAC TGGTATCGAT ATCGTGGCTG CACAGATCCA GATTGCTGCT
G A S L E Q L G L T Q D R I S A R G F A
1081 GGTGCAAGCC TCGAGCAACT GGGCCTGACT CAGGACCGCA TCTCCGCCCC CGGATTTGCC
I Q C R I T T E D P A K G F S P D T G K
1141 ATTCAATGTC GTATCACCAC GGAAGATCCC GCCAAGGGGT TCTCTCCGGA TACTGGTAAG
I E V Y R S A G G N G V R L D G G N G F
1201 ATTGAGGTTT ATCGTTCCGC TGGTGGTAAC GGTGTCCGTC TGGATGGTGG TAACGGTTTC
A G A I I T P H Y D S M L V K C T C R G
1261 GCTGGTGCTA TCATACCCCC TCACTACGAC TCCATGCTGG TCAAGTGCAC CTGCCGTGGT
S T Y E I A R R K V V R A L V E F R I R
1321 TCGACCTATG AAATCGCTCG TCGCAAGGTT GTGCGTGCCT TGGTCGAGTT CCGTATTCGT
G V K T N I P F L T S L L S H P T F V D
1381 GGTGTGAAGA CCAACATTCC CTTCTGACT TCGCTTCTGA GCCACCCGAC CTTTCGTCGAT
G N C W T T F I D D T P E L F S L V G S
1441 GGAAACTGCT GGACCACTTT CATCGACGAC ACCCCTGAAT TGTTCTCTCT TGTCGGCAGT
Q N R A Q K L L A Y L G D V A V N G S S
1501 CAGAACCGTG CCCAGAAGCT GCTCGCATAC CTCGGCGATG TAGCTGTCAA CGGTAGTAGC
I K G Q I G E P K L K G D V I K P K L F
1561 ATCAAGGGCC AAATTGGCGA GCCCAAGCTC AAGGGTGATG TCATCAAGCC GAAGCTTTTC
D A E G K P L D V S A P C T K G W K Q I
1621 GATGCCGAGG GCAAGCCGCT TGACGTTTCC GCCCCCTGCA CCAAGGGTTG GAAGCAGATT
L D R E G P A A F A K A V R A N K G C L
1681 CTGGACCGGG AGGGTCCGGC TGCCTTTGCG AAGGCCGTGC GTGCCAACAA GGGTTGCTTG
I M D T T W R D A H Q S L L A T R V R T
1741 ATCATGGATA CTACCTGGCG TGACGCCAC CAGTCTTTGC TGGCCACCCG TGTGCGTACC
I D L L N I A H E T S Y A Y S N A Y S L

Fig. 8A

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1801 ATCGACTTGT TGAACATCGC CCATGAGACC AGCTACGCCT ACTCCAATGC GTACAGTTTG
E C W G G A T F D V A M R F L Y E D P W
1861 GAATGCTGGG GTGGTGCTAC CTTCGATGTG GCCATGCGTT TCCTCTATGA GGACCCCTGG
D R L R K M R K A V P N I P F Q M L L R
1921 GACCGCCTGC GCAAGATGCG TAAGGCTGTT CCTAACATCC CATTCCAGAT GTTGCTCCGT
G A N G V A Y S S L P D N A I Y H F C K
1981 GGTGCCAACG GTGTGCCTA CTCTTCCCTC CCAGACAACG CCATCTACCA CTTCTGTAAG
Q A K K C G V D I F R V F D A L N D V D
2041 CAGGCTAAGA AGTGCGGTGT CGACATTTTC CGTGTTCG ACGCCCTCAA CGATGTCGAT
Q L E V G I K A V H A A E G V V E A T M
2101 CAGCTCGAGG TCGGTATCAA GGCTGTTTCAT GCTGCCGAGG GTGTTGTCGA GGCCACCATG
C Y S G D M L N P H K K Y N L E Y Y M A
2161 TGCTACAGCG GTGACATGCT GAACCCCCAC AAGAAGTACA ACCTGGAGTA CTACATGGCC
L V D K I V A M K P H I L G I K D M A G
2221 TTGGTGGATA AGATTGTAGC CATGAAGCCT CACATCCTTG GTATCAAGGA TATGGCCGGT
V L K P Q A A R L L V G S I R Q R Y P D
2281 GTGCTGAAGC CCCAGGCCGC TCGCCTGTTG GTGGGCTCCA TCCGTCAGCG CTACCCTGAC
L P I H V H T H D S A G T G V A S M I A
2341 CTTCCCATCC ACGTCCACAC CCACGACTCC GCTGGTACTG GTGTAGCTTC CATGATTGCC
C A Q A G A D A V D A A T D S M S G M T
2401 TGTGCCCAGG CGGGTGCCGA CGCCGTGGAC GCCGCGACCG ACAGCATGTC CGGTATGACC
S Q P S I G A I L A S L E G T E Q D P G
2461 TCCCAGCCTA GCATTGGTGC CATTCTGGCC TCTCTTGAGG GCACTGAGCA AGACCCCGGT
L N L A H V R A I D S Y W A Q L R L L Y
2521 CTCAACCTCG CCCACGTGCG CGCTATTGAT AGCTACTGGG CACAGCTGCG CTTGCTCTAC
S P F E A G L T G P D P E V Y E H E I P
2581 TCTCCTTTCG AGGCGGGTCT CACTGGCCCC GACCCTGAGG TCTACGAGCA CGAGATCCCT
G G Q L T N L I F Q A S Q L G L G Q Q W
2641 GGTGGTCAGT TGACCAACCT TATCTTCCAG GCCAGTCAGC TCGGCTTGGG CCAGCAGTGG
A E T K K A Y E A A N D L L G D I V K V
2701 GCCGAAACCA AGAAGGCCTA TGAGGCGGCT AATGATTTAC TCGGCGACAT TGTAAGGTC
T P T S K V V G D L A Q F M V S N K L T
2761 ACTCCACCT CCAAGGTGGT CGGTGACTTG GCTCAGTTCA TGGTCTCGAA CAACTGACT
P E D V V E R A G E L D F P G S V L E F
2821 CCAGAGGATG TTGTTGAGCG TGCTGGTGAG CTGGACTTCC CTGGTTCTGT GCTCGAATTC
L E G L M G Q P F G G F P E P L R S R A
2881 CTCGAAGGTC TCATGGGACA GCCCTTCGGT GGATTCCCCG AGCCATTGCG CTCCCGCGCC
L R D R R K L E K R P G L Y L E P L D L
2941 CTGCGCGATC GCCGCAAGCT CGAGAAGCGT CCAGGTCTCT ACCTCGAGCC TTTGGATTTC
A K I K S Q I R E K F G A A T E Y D V A
3001 GCTAAGATCA AGAGCCAGAT CCGTGAGAAG TTCGGTGCTG CTACTGAGTA TGACGTGGCC
S Y A M Y P K V F E D Y K K F V Q K F G
3061 AGCTATGCCA TGTATCCCAA GGTCTTCGAG GACTACAAGA AGTTCGTCCA GAAGTTCGGT
D L S V L P T R Y F L A K P E I G E E F
3121 GATCTCTCCG TCTTGCCAC ACGGTACTTC TTGGCCAAGC CTGAGATTGG CGAGGAGTTC
H V E L E K G K V L I L K L L A I G P L
3181 CACGTTGAGC TGGAGAAGGG TAAGGTGCTC ATCCTGAAGT TGTTGGCCAT CGGCCCTCTT
S E Q T G Q R E V F Y E V N G E V R Q V
3241 TCAGAGCAGA CTGGTCAGCG TGAGGTCTTC TACGAAGTCA ACGGTGAGGT GCGCCAGGTC
A V D D N K A S V D N T S R P K A D V G
3301 GCTGTTGATG ACAACAAGGC TTCCGTGGAC AACACTTCAC GCCCTAAGGC CGATGTGGGT
D S S Q V G A P M S G V V V E I R V H D
3361 GACAGCAGCC AGGTCCGTGC TCCTATGAGC GGTGTGGTTG TTGAAATCCG TGTCCACGAT
G L E V K K G D P L A V L S A M K M
3421 GGTCTGGAGG TTAAGAAGGG TGACCCACTT GCCGTCCTGA GTGCCATGAA GATGGTAAGT
E M
3481 TCATTCCGAA TCATTTTCT CACTGGTCAA CTACAGATGC TAACAGCTTA TCCAGGAAAT
• V I S A P H S G K V S S L L V K E G D S •
3541 GGTTATCTCT GCTCCTCACA GTGGAAAGGT CTCCAGCTTG CTGGTCAAGG AGGGCGATTC
• V D G Q D L V C K I V K A *
3601 TGTGGATGGC CAGGATCTCG TCTGCAAGAT CGTCAAAGCG TAA

Fig. 8B

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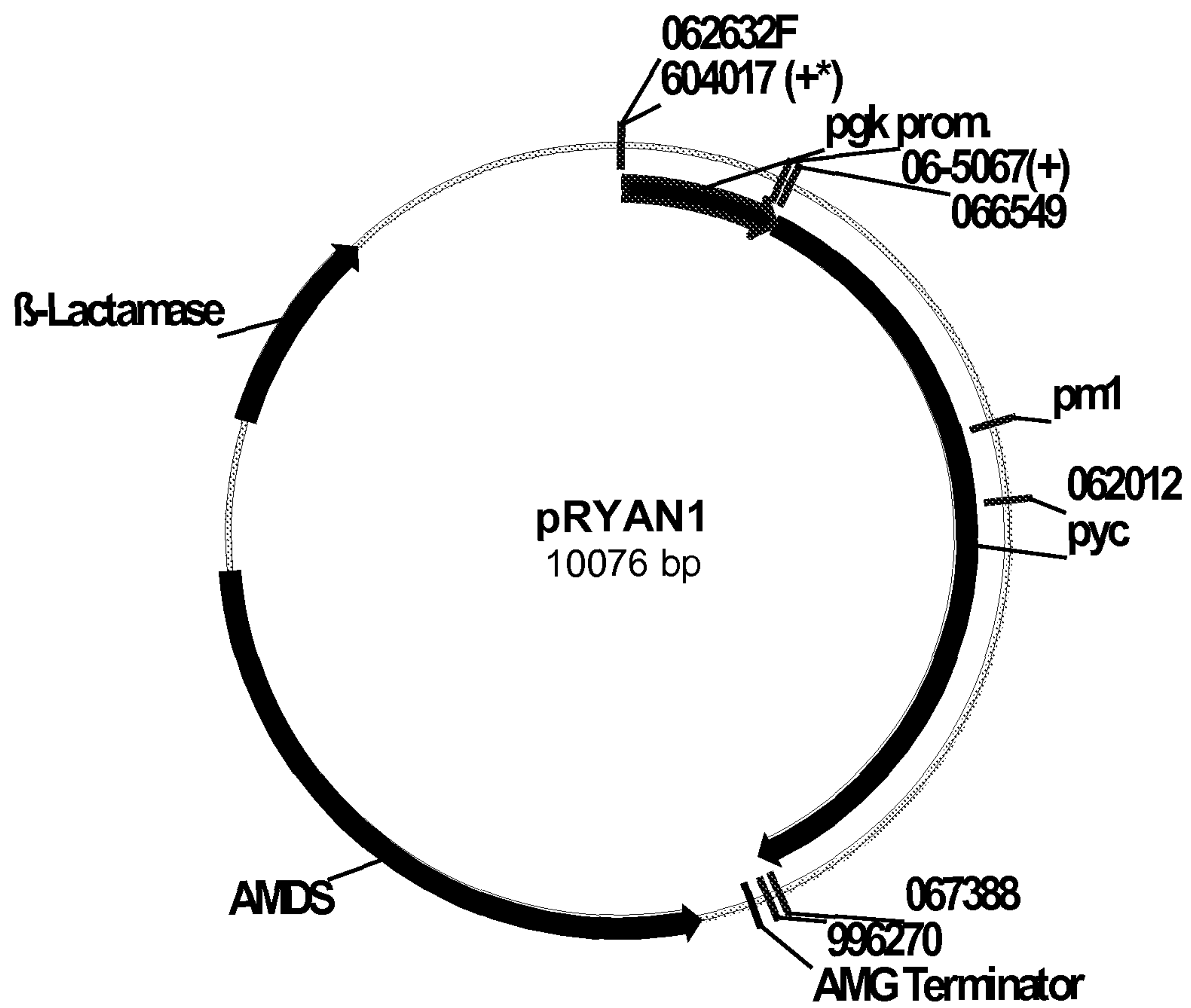


Fig. 9

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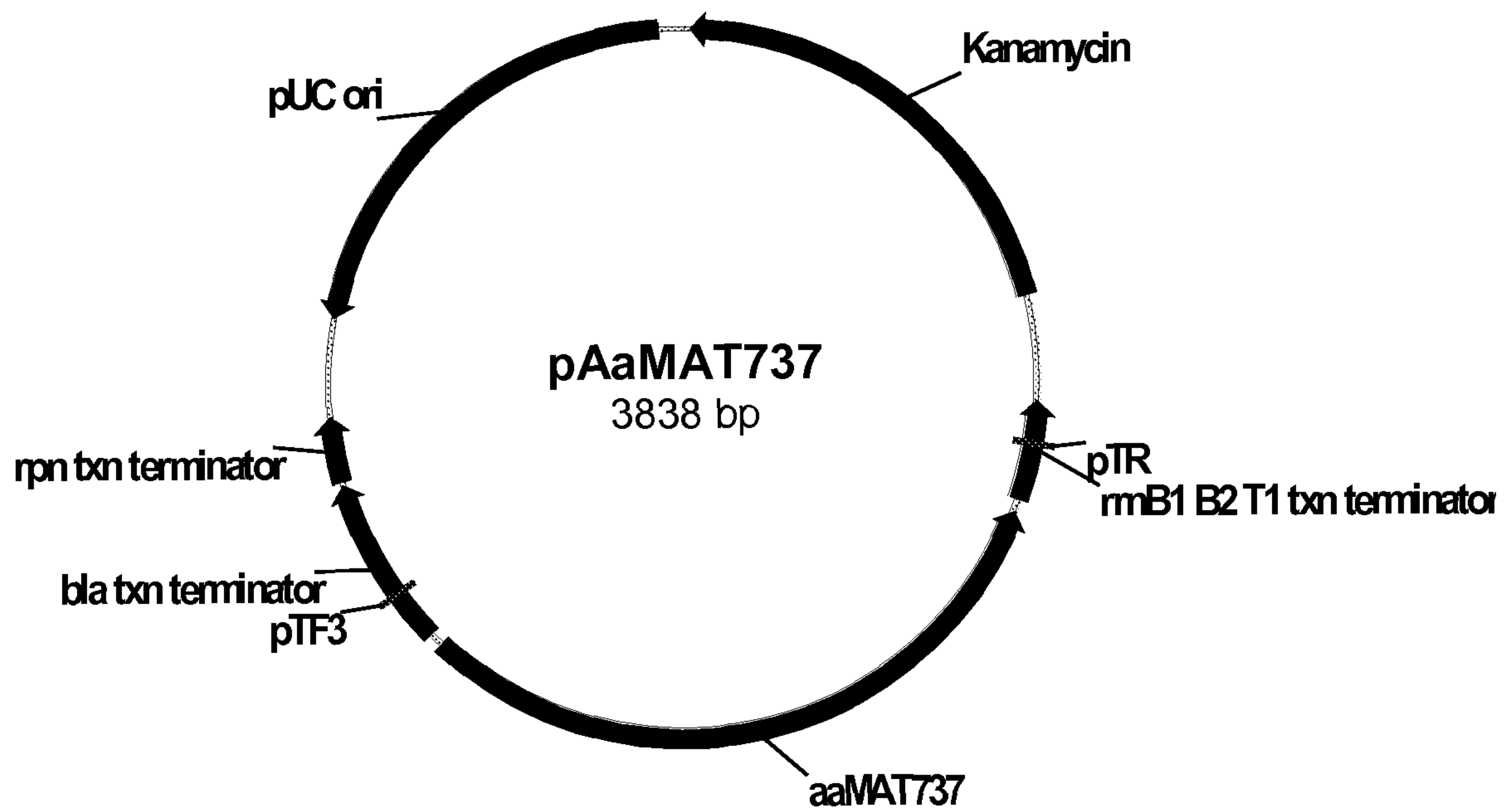


Fig. 10

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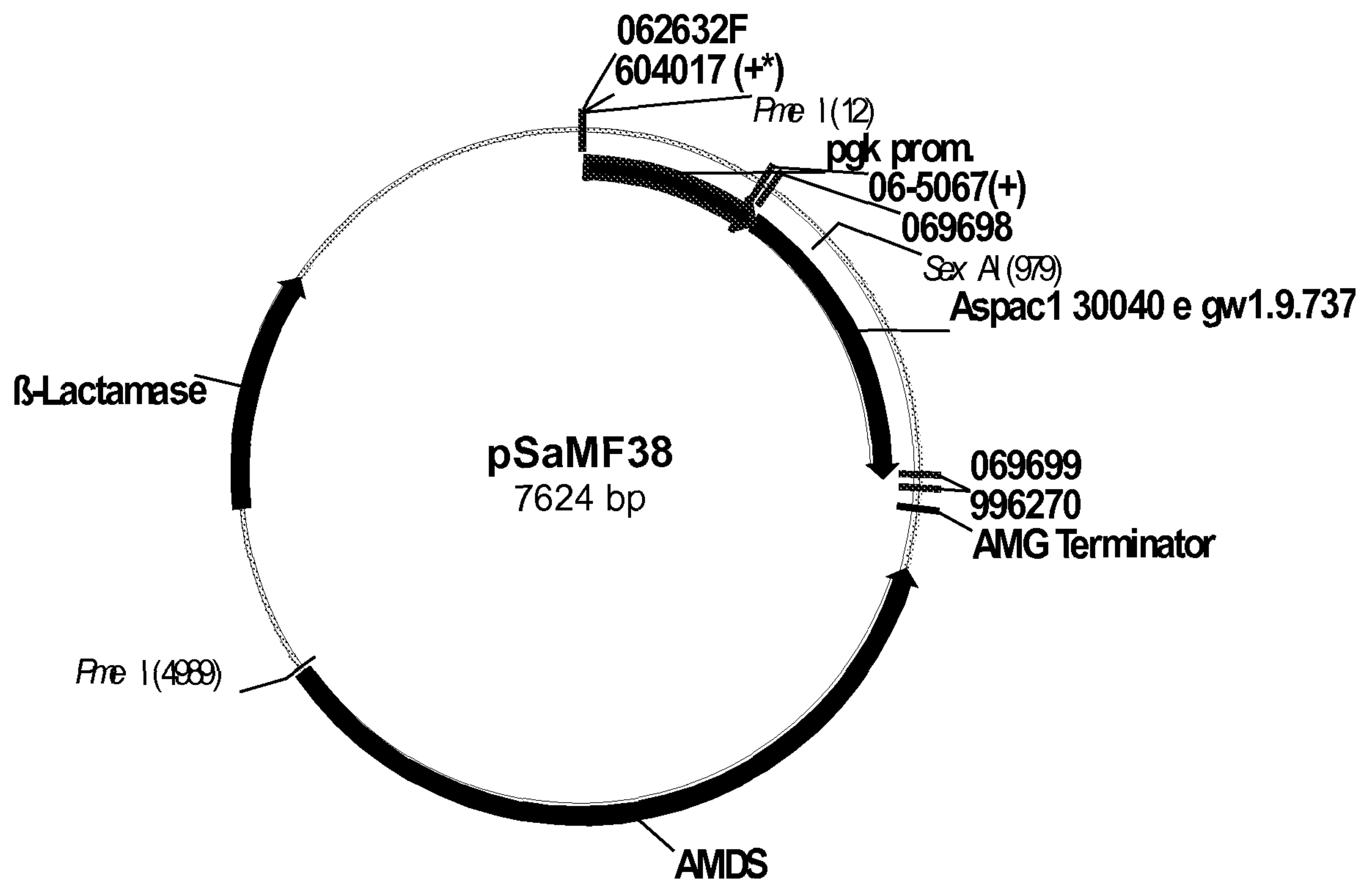


Fig. 11

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      M L G Q H S P P G T S C S D L T T Y Q H E L K A
1  ATGCTCGGGCAACACTCGCCTCCCGGCACCTCCTGCTCGGACCTTACAACATACCAACATGAGCTTAAAGCC
      S K Y S S S T N V S L R D R L R H F T W A W Y T
73  TCCAAATACTCTAGTTCCACCAATGTGTCTCTACGGGACCGTCTGCGTCATTTTACCTGGGCCTGGTATACT
      L T M S T G G L A L L L A S Q P Y T F S G L Q Q
145  CTGACTATGAGCACCGGCGGCCTAGCGCTTCTGCTGGCCAGCCAGCCCTACACCTTCTCCGGA CTGCAACAG
      I G L A V Y I I N L V F F A L L C S L M A T R F
217  ATCGGGCTTG CAGTCTATATCATCAACCTGGTCTTCTTTGCTTTGCTGTGCAGCCTCATGGCCACGCGCTTC
      I L H G N F L D S L R H D R E G L F F P T F W L
289  ATTCTCCACGGCAACTTCCTCGACTCCCTCCGACACGACCGCGAGGGTCTTTTCTTTCCCACTTTCTGGCTT
      S I A T I I T G L Y R Y F G D T T Q P A F I Y A
361  TCCATTGCAACTATCATCACCGGACTCTACCGCTACTTCGGCGACACCACACAGCCTGCATTCATTTACGCC
      L E V L F W L Y C A F T L M T A I I Q Y S F V F
433  CTTGAGGTGCTTTTCTGGCTCTACTGTGCCTTCACACTGATGACCGCTATCATCCAATACTCTTTTGTCTTT
      T A H H Y P L Q T M M P S W I L P A F P I M L S
505  ACTGCCCACCACTACCCTCTACAAACGATGATGCCCTCGTGGATCCTCCCCGCATTCCCCATCATGCTAAGC
      G T I A S V I A E Q Q P A R S A I P M I V A G T
577  GGCACGATCGCCTCTGTCATTGCCGAACAGCAGCCCGCGCGCTCTGCTATTCCCATGATCGTCGCCGGCACC
      T F Q G L G F S I S F L M Y A H Y I G R L M E T
649  ACCTTCCAAGGCCTTGGCTTCTCCATCAGTTTCTCATGTACGCGCACTATATCGGACGCCTCATGGAGACG
      G L P S R E H R P G M F I C V G P P A F T A L A
721  GGCCTTCCGTCCCGGGAACACCGACCCGGGATGTTTCATCTGCGTTGGCCCCCCTGCTTTCACGGCCCTTGCC
      L I G M T N G L P E D F Q V L Q D P H P F Q D A
793  CTAATCGGCATGACCAACGGCCTTCTGAGGATTTTCAAGTCCTTCAAGACCCGCACCCCTTTCAAGACGCG
      H I L R L L A I A T G A F L W A L S L W F F S I
865  CATATCCTCCGACTCCTTGCCATCGCCACGGGCGCCTTCCTCTGGGCCCTCAGTCTCTGGTTCTTCAGCATT
      A I I A T I R L P P T A F H L N W W A M V F P N
937  GCCATTATCGCCACCATCCGCCTCCCACCTACGGCCTTCCACCTCAACTGGTGGGCCATGGTTTTTCAAAC
      T G F T L A T I T L G K A F D S P G V K G V G S
1009  ACGGGTTTTACTCTCGCGACCATCACGCTGGGCAAAGCCTTCGATAGCCCTGGAGTCAAGGGCGTCGGATCT
      A M S I C I V G M W L F V F A S N I R A V V K R
1081  GCCATGTCCATTTGCATCGTGGGGATGTGGCTGTTGCGAGCAATATCCGCGCCGTTGTCAAACGG
      D I V F P G K D E D V S E *
1153  GATATTGTGTTTCTGGCAAGGACGAGGATGTATCGGAGTAA
```

Fig. 12

	M	L	G	Q	H	P	P	P	D	T	S	C	S	D	L	T	T	Y	Q	H
1	ATG	CTC	GGGC	AAC	ATC	CGCC	TCCC	GAC	ACC	TCCT	GCT	CGG	ACCT	TACA	AAC	ATAC	CAG	CAT		
	E	L	K	A	S	K	Y	S	S	S	T	N	V	S	L	R	D	R	L	R
61	GAG	CTC	AAAG	CCT	CCA	AATA	CTCT	AGT	TCC	ACCA	ATG	TGT	CTCT	ACG	GGGA	CCGT	CTG	CGT		
	H	F	T	W	A	W	Y	T	L	T	M	S	T	G	G	L	A	L	L	L
121	CAT	TTT	ACCT	GGG	CGT	GGTA	TACT	CTG	ACT	ATG	AGC	ACCG	GCG	GTCT	AGC	CCT	CCT	GCT	G	
	A	S	Q	P	Y	S	F	S	G	L	Q	Q	I	G	L	A	V	Y	I	I
181	GCC	AGC	CAGC	CCT	ACT	CCTT	CTCC	GGAC	TG	CAAC	AGAT	CG	GGCT	TGC	AGT	CTAC	ATC	ATC		
	N	L	A	F	F	A	L	L	C	S	L	M	A	A	R	F	I	L	H	G
241	AAC	CTG	GCCT	TCT	TTG	CGTT	GCT	GTG	TAGC	CTCA	TGG	CCG	CAC	GCTT	CAT	TCT	CCAC	GGC		
	N	F	L	D	S	L	R	H	D	R	E	G	L	F	F	P	T	F	W	L
301	AACT	TCCT	CG	ACT	CCCT	CCG	ACAC	GAC	CCGC	GAGG	GTCT	TTT	TCT	TTT	CCT	AC	TTT	CTG	GCT	T
	S	I	A	T	I	I	T	G	L	Y	R	Y	F	G	D	T	T	Q	P	A
361	TCT	ATT	GCAA	CTAT	CAT	CAC	CGGC	CTG	TAC	CGCT	ACTT	CG	GCG	ACAC	CAC	ACAG	CCT	GCA		
	F	I	Y	A	L	E	V	L	F	W	L	Y	C	A	F	T	L	M	T	A
421	TTC	ATTT	ACG	CTCT	TG	AGGT	GCT	CTT	CTGG	CTCT	ACT	GTG	CCTT	CACT	CT	GAT	GAC	CGCT		
	I	I	Q	Y	S	F	V	F	T	A	H	H	Y	P	L	Q	T	M	M	P
481	ATT	ATC	CAAT	ACT	CCT	TTTGT	CTTT	ACC	GCC	CACC	ACT	ACC	CTCT	ACAA	AAC	GAT	GAT	GCCC		
	S	W	I	L	P	A	F	P	I	M	L	S	G	T	I	A	S	V	I	A
541	TCAT	GGAT	CC	TCCC	CG	CATT	CCCT	ATC	ATG	CTC	AGC	GGCA	CGAT	CGC	CTC	CGTC	ATT	GCC		
	E	Q	Q	P	A	R	S	A	I	P	M	I	V	A	G	T	T	F	Q	G
601	GAAC	AGC	AGC	CCG	CGC	GCTC	TGCT	ATT	CCC	ATGA	TCGT	CG	CCGG	CACC	CAC	CTT	CCA	AGGC		
	L	G	F	S	I	S	F	L	M	Y	A	H	Y	I	G	R	L	M	E	T
661	CTT	GGCT	TTCT	CCAT	CAG	TTT	CCT	CAT	GTAC	GCG	CACT	ATA	TCG	GGC	GGCT	CAT	GGAG	ACG		
	G	L	P	S	R	E	H	R	P	G	M	F	I	C	V	G	P	P	A	F
721	GGC	CTT	CCGT	CCG	GGA	ACA	CCG	ACCC	GGG	ATGT	T	CAT	CT	GCG	TTGG	CCC	CCCG	GCTT	T	C
	T	A	L	A	L	I	G	M	T	N	G	L	P	E	D	F	Q	V	L	Q
781	ACG	GCC	CCTTG	CCCT	AAT	CGG	CAT	GAC	CAAC	GGC	CTT	CCTG	AGG	ATTTT	TCA	AGT	CCTT	CAA		
	D	P	H	P	F	Q	D	A	H	I	L	R	L	L	A	I	A	T	G	A
841	GAC	CCG	CACC	CCTT	TCA	AGA	CGCG	CAC	ATC	CTCC	GACT	CC	TTG	CCAT	CGC	CAC	GGG	CGCC		
	F	L	W	A	L	S	L	W	F	F	S	I	A	I	I	A	T	I	R	L
901	TTC	CTC	TGGG	CCCT	CAG	TCT	CTGG	T	TCTTT	AGCA	TTG	CCA	TCAT	CGCC	CAC	CAT	CCG	CCTC		
	P	P	T	A	F	H	L													